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THE DEVELOPMENT OF EIMERIA BRUNETTI LEVINE IN THE DIGESTIVE TRACT OF CHICKENS[1]

Janet I. Boles and Elery R. Becker

Department of Zoology and Entomology, Iowa State College, Ames, Iowa

Eight species of coccidia of the genus Eimeria are known to parasitize the digestive tract of the common fowl. The developmental cycles of six of them, E. tenella, E. mitis, E. acervulina, E. maxima, E. necatrix, and E. praecox, were delineated by Tyzzer (1929) and Tyzzer, Theiler, and Jones (1932). The intestinal phases of E. hagani Levine, 1938, and E. brunetti Levine, 1942, however, have not been depicted. The present paper is concerned with E. brunetti, originally discovered by Levine (1938) in an outbreak of coccidiosis, and subsequently again noted by him (1943, 1945). The "reproductive potential" of E. brunetti and certain other avian species was studied by Brackett and Bliznick (1952).

MATERIALS AND METHODS

The hosts were New Hampshire Red chicks obtained from a hatchery when one day old, and kept coccidium-free until experimentally infected. The parasite was a line of E. brunetti descended from a single oocyst isolated from the progeny of an infection found on a farm in central Iowa. The oocysts were permitted to sporulate in a shallow layer of 2 per cent solution of potassium dichromate in a Petri dish. The number of infective oocysts employed in the experimental infections usually varied from 100,000 to 900,000. The lack of success in locating sporozoites very early in the infection led to administering to one chicken three doses of six million sporulated oocysts at hourly intervals, and the killing of the bird one hour after the last inoculation. Fresh smears of intestinal walls of all infected chicks were studied microscopically. Permanent stained smears were also made of the intestinal content and wall scrapings in certain cases.

Pieces of the digestive tract were removed from chickens sacrificed at various time intervals after inoculation. These were fixed in formalin-Zenker's (Bensley and Bensley, 1938), washed, dehydrated, embedded in paraffin, sectioned at 5.0 microns, and stained in either iron-haematoxylin or Giemsa. The Giemsa stain was a modification of McNamara's stain according to Shortt and Cooper (1948), and further modified by Wolcott (1952). Tissue smears were fixed in formalin-Zenker's and stained in Giemsa only. The Giemsa stain was the more useful of the two.

Measurements were made with the aid of a calibrated camera lucida. Frequent reference was made to Calhoun's (1933) work on the microscopic anatomy of the chicken's intestine.

[1]This study was supported, in part, by a grant from the Lederle Laboratories Division, American Cyanamid Company.

RESULTS

E. brunetti was found to parasitize the epithelium of the villi in contact with or close to the basement membrane. Invasion of the cores of the villi was observed in certain heavy infections. A few schizonts developed in the gland cells. Penetration of the sporozoites and the development of the first generation merozoites occurred mostly in the small intestine from the upper to the lower part, but subsequent developmental stages were observed throughout the gut from the middle small intestine to the cloaca.

Excystation of oocysts. Sporozoites could not be found in the intestines of four chickens that received less than a million sporulated oocysts. Sporulated oocysts, however, were found from the crop to the gizzard.

The crop, proventriculus and gizzard of a chicken inoculated with ten million oocysts contained sporulated oocysts and spores holding sporozoites, but no liberated sporozoites were observed in those places. Pratt (1937) reported that the sporozoites of E. tenella were released in the crop.

Many broken and empty oocysts were observed throughout the gut. Empty spores escaped from oocysts and failure to find empty spores still inside oocyst walls indicated that the sporozoites escaped from released spores. Fig. 2 shows the typical empty sporocyst with an intact Stieda body and a large rent in its side.

Sporozoites, observed in fresh smears of the upper, middle and lower small intestine and, less frequently, in the rectum and caeca, showed flexion movements of the more tapering end (Fig. 3), but no movement of translation. The pressure of the cover glass may have been responsible.

In stained tissue sections, sporozoites were observed in the epithelial cells of the upper, middle, and lower small intestine, and in the rectum. Many sporozoites also invaded the core of the villus (Fig. 20, 3 hrs.). Tyzzer, Theiler, and Jones (1932) stated that about as many sporozoites of E. praecox passed through the epithelium of the villus as established themselves there, and considered it probable that those in the tunica propria did not undergo further development. The same statements would probably hold for E. brunetti, because the later stages were located in the apithelium.

The size of the sporozoites varied greatly. Some sporozoites were long and narrow and had two refractile globules which stained pink with Giemsa (Fig. 3). Others were long and narrow, but contained only one refractile globule. Others, measuring on the average 6.7 by 2.8 microns (Fig. 4), were shorter and contained one refractile globule. All kinds were found in tissue smears from the middle and lower small intestine at the time the chicken was killed. The upper small intestine at that time only showed the long kind with two refractile globules. Only the short kind with only one globule was observed in the stained sections. Roudabush (1937) stated that in the case of E. nieschulzi the two globules of the sporozoite evidently united after the sporozoite entered the cell. From the foregoing observations it appears that either the same event occurs in E. brunetti or the two globules unite before they enter the cell. The sporozoites still within the spores of the oocysts show two globules.

A zinc sulfate flotation was made on feces collected from beneath the

cage holding the heavily infected chicken until it was killed. The surface film contained unsporulated, incompletely sporulated, and completely sporulated oocysts. Sporulated oocysts were also observed in fresh smears from the content of the gut throughout its length. These observations confirmed Tyzzer's (1929) observation that a variable number of oocysts of Eimeria pass through the intestine unhatched, and that the number ingested furnished only a rough index to the actual dosage.

Asexual stages. Parasites were not recognized in the tissue from the time sporozoites were noted at three hours until the second day (51 hours), when developing generation I schizonts occurred in the upper and lower small intestine and caeca, particularly the proximal portions. These were formed from sporozoites, and were distinguishable by the refractile globule (center of Fig. 5) which evidently was the residuum of the refractile globules of the sporozoite. This globule disappeared as development proceeded. The mature generation I schizonts (Fig. 6) were very large, measuring approximately 20 by 30 microns in tissue sections, and contained approximately 200 merozoites. The morphology of the merozoites was indistinct. Fig. 7 is a merozoite drawn from a tissue section of a generation I schizont. These schizonts were found in the epithelium lying close to the basement membrane along the side of the villus. Fig. 20 shows the schizonts in relation to the villus of the upper small intestine.

At the same time (51 hours) that generation I schizonts were found, merozoites that had just entered cells were observed in the lower small intestine, the narrow tubular portion of the caeca, and the rectum (Fig. 21). Those were the only stages found in the rectum at that time. Those merozoites must have been generation I merozoites that had been released from schizonts. Although only a few generation I schizonts with merozoites were seen, they must have been fairly numerous in some part of the intestine that was not sectioned, such as the middle small intestine which was not taken at that time. The merozoites had to come from generation I schizonts.

At 60 hours, the generation I schizont was the only form found in the upper small intestine (Fig. 20). The infection, however, was mostly made up of generation I merozoites that had just entered cells (Fig. 8) and others that had started to divide to form generation II schizonts (Fig. 9). These developing generation II schizonts were found primarily in the lower small intestine and narrow, tubular portion of caeca of this chicken (Fig. 21).

The chicken killed in the early afternoon of the third day (76 hours) may have been resistant, or the tissue not taken at the proper places to observe the infection. Very few parasites were observed. The middle small intestine had several generation I schizonts (Fig. 21), but the rest of the gut did not show parasites, except the narrow, tubular portion of the caeca. That portion of the caeca had a heavy infection of merozoites which had just entered epithelial cells, and others more advanced with multiple nuclei.

The chicken killed on the evening of the third day (82 hours) had a heavy infection of merozoites which had just entered cells, and others that had become schizonts with a few multiple nuclei (Fig. 22). There were a few with merozoites already formed. These schizonts were not as large as the generation I schizonts, and were taken to be generation II schizonts.

At that time parasites were first observed in the cloaca. None could be found in the upper small intestine.

On the morning of the fourth day (95 hours), two types of schizonts, large and small, were observed. The large ones averaged 20.9 by 16.2 microns, and contained 50 to 60 merozoites (Figs. 11 and 12). The smaller ones, that were almost round, averaged 9.8 by 8.8 microns, and contained approximately 12 to 14 merozoites. The latter merozoites appeared longer and more pointed (Fig. 10).

Since the large developing schizonts first appeared on the evening of the third day (82 hours), 13 hours prior to the time both types were found (95 hours), they should be considered generation II schizonts. No definite decision was made in regard to the small schizonts. The size difference could have been caused by crowding, or there could have been sexual dimorphism, with one size developing into macrogametocytes and the other into microgametocytes. A third possibility is that these small schizonts were a third generation.

The infection at 95 hours was very heavy in the lower small intestine and rectum, while the middle small intestine and narrow, tubular portion of the caeca were moderately infected. Both of the latter had a majority of large schizonts. Two pieces of the lower small intestine were taken, one of which was very heavily infected and the other apparently not infected. The infection must have been spotty. The portion closest to the caeca was the only part that showed pin-point lesions at the time the tissue was taken, and thus was probably the infected portion. Fig. 25, taken of the rectum, shows developing large schizonts in the epithelium resting on the basement membrane, and numerous schizonts, mostly of the small variety, in the tip of the villus. Where the infection was heavy, schizonts shallowly invaded the tunica propria and the glands, otherwise they were confined to the epithelium of the villi.

Sexual stages. Very young microgametocytes were not recognized. Microgametocytes were formed from merozoites, of course, and after a merozoite had entered a cell and rounded up, it was impossible to tell whether it was fated to be a macrogametocyte or a microgametocyte until the nucleus divided or it was apparent by its size that the nucleus was not going to divide. Since the nucleus of the macrogametocyte does not divide, a cell with more than one nucleus could not be a macrogametocyte. Schizonts, like microgametocytes, however, also had multiple nuclei, but their nuclei were larger and the karyosomes more prominent than those of the microgametocytes. The nuclei of macrogametocytes appeared to have very faint karyosomes, or none at all, and thus could be distinguished from schizonts. As development proceeded, the microgametocytes grew in size and number of nuclei.

Microgametocytes were first recognized on the fifth day. They appeared as large cells with many nuclei. Fig. 27 shows a large developing microgametocyte lying next to a mature microgametocyte. As development proceeded, faint lines of cleavage appeared (Fig. 30). Next, the nuclei became prominent, dark and oblong, and the cell continued to show faint lines of cleavage (Fig. 16). These prominent nuclei were probably aggregates of chromation. Cleavage became more prominent and the nuclei became thinner and U-shaped as development proceeded (Fig. 17). The cell seemed to increase in size, and the nuclei which had been U-

shaped elongated and thickened until they were comma-like (Fig. 18). These elongated more until they became mature male gametes which developed in whorls around residual masses (Figs. 19, 27 and 28). This multicentric arrangement is said to occur in E. maxima also (Tyzzer, 1929) but in no other form of chicken coccidia. In both species the microgametocytes were fewer in number than macrogametocytes. Flagella were not observed on microgametes in tissue sections or smears, but failure to observe them is by no means an indication that they were absent. Young macrogametocytes were first distinguishable on the evening of the fourth day (106 hours) throughout the gut, excluding the upper small intestine and bursa cloaca. They were spherical cells containing a relatively large homogenous nucleus with a large karyosome staining a dull red. The cytoplasm had many small granules so arranged that it appeared spongy (Fig. 13). These young macrogametocytes were not measured, since age would cause such great variation. Fig. 29 shows many young macrogametocytes.

On the fifth and sixth days mature macrogametocytes were evident. They were oval, and their size varied greatly depending on whether they were measured from smears or sections. In smears, they were larger, averaging 25.2 by 22.2 microns, while in sections they measured on the average 18.3 by 10.9 microns. The nucleus was ellipsoidal with a large karyosome. The nuclear membrane was obscured by granules that formed a peripheral layer around the nucleus. The cytoplasm appeared spongy, and around the periphery were bright red "plastic granules" that later contributed (apparently) to the formation of the oocyst wall (Fig. 14). (Roudabush, 1937 and Goodrich, 1944). Actual fertilization by a microgamete was not observed.

Macrogametocytes that were developing into oocysts had a denser peripheral layer of granules around the nucleus and a karyosome diminished in size. The outer wall that was formed by the plastic granules stained red, as had the plastic granules. The cytoplasm appeared more granular than it did as a macrogametocyte, and thus formed a closer spongy network. There was some indication of residual small, very lightly pink-staining granules around the periphery of the cell approximately where the plastic granules had been located. They were somewhat hard to see (Fig. 15). Fig. 30 shows a late-developing oocyst with a thin wall. It is among developing microgametocytes which show the aforementioned clefts.

Levine (1942) described the oocysts as being egg-shaped or oval, with an average length of 26.8 microns and average width of 21.7 microns. This agreed very closely with the size of macrogametocytes in fresh and stained smears. He stated that the narrow end of the oocyst was rounded and usually contained a refractile body in sporulated oocysts. This was probably the polar inclusion. Ten per cent of his oocysts contained 2, or 3, polar inclusions located close to one another. Out of 111 oocysts observed in this study, 70 per cent had one, 25 per cent had two, 3 per cent had three, and 2 per cent had four separable polar inclusions. The wall of the oocyst was thinner at the narrow end. The spore had a Stieda body, and an intraresidual body (Fig. 1).

The development of macrogametocytes, microgametocytes and oocysts occurred primarily in the lower small intestine, caeca, rectum, and

cloaca where they rested on the basement membrane, and thus were below the cell nuclei (Figs. 23, 27, 28, 29, 30).

Figs. 20, 21, 22, and 23 give a composite picture of the stages found in the tissues at the various hours. Fig. 24 shows the distribution of E. brunetti in the intestine.

Pathology

Gross changes first appeared on the evening of the third day (82 hours), when the infected chickens became listless or sick. Red pin-point lesions were observed in the upper, middle, and lower small intestine. They were most numerous in the lower small intestine for about three inches directly above the caeca. Tissue sections showed a very heavy infection with young schizonts, some eosinophilia, some hyperemia, and some sloughing of the epithelium.

On the morning of the fourth day (95 hours), the host became very sick. It was listless and its feathers were ruffled. The intestine was whitish colored. Pin-point lesions were observed in the portion of the lower small intestine closest to the caeca, and tissue taken from there was heavily charged with parasites, while tissue taken a few inches higher showed no parasites. Fig. 25, taken of the rectum of this chicken, shows the very heavy infection of the epithelium with sloughing of the infected epithelium and hyperemia. Tips of the villi were more heavily infected than the sides, and in very heavily infected villi the tunica propria was invaded to some extent as well as the gland epithelium.

Whitish feces of a liquid consistency tinged with blood were passed by the chicken killed the evening of the fourth day (106 hours). Hordes of merozoites had been released, and the sexual stages were beginning. The middle and lower small intestine had pin-point lesions, and the lumen contained shreds of salmon-colored material which probably represented sloughed mucosa. The pin-point lesions of the lower small intestine became more numerous toward the caeca. The narrow, tubular portion of the caecum contained pin-point lesions. The dilated portion was plugged with what appeared to be dehydrated normal caecal contents in combination with dry clotted blood. A small amount of blood and a few pin-point lesions were visible. Tyzzer, Theiler, and Jones (1932) noted a similar condition in the case of infection with E. necatrix and postulated that the drying out was due to failure in the supply of fluid normally furnished by the small intestine. Levine (1942) stated that a caseous core a few millimeters long was present in the narrow, tubular portion of the caecum of a chicken infected with E. brunetti. It may have been in our material, but overlooked, since it probably was continuous with the dried content. Something must have prevented the normal flow of fluid into the caecum. Fig. 26, taken of the dilated portion of the caecum, shows the damage done by the plug. A homogeneous colloidal discharge, which stained pink with Giemsa, is visible in the glands, and the same discharge mixed with numerous bacteria is visible surrounding the villi. There was hyperemia, some hemorrhage, and sloughing of the epithelium in this area also, but very few parasites.

The general appearance of the chicken killed the morning of the fifth day (120 hours) did not indicate it was sick. The lower small intestine

had pin-point lesions which were more numerous in the area six to three inches above the caeca. The rectum and caecum also had pin-point lesions, and the caecum contained a watery pink-colored fluid that was blood-tinged. The whole intestine appeared swollen.

Pin-point lesions in the lower small intestine, in the caecum at its juncture with the intestine, and in the rectum were observed in the chicken killed on the sixth day. The pin-point lesions of the lower small intestine appeared more brown than red, and extended throughout the lower small intestine. Fig. 31 shows an area of the small intestine where the villi were denuded of epithelium and slightly hyperemic. Only the basement membrane separated the tunica propria from the lumen. In other areas of the small intestine, the epithelium was intact although it was heavily infected with sexual forms. The severe epithelial denudation of the villi most probably was caused by the asexual stage rather than the sexual stage of the parasite, although it cannot be stated for certain. The chickens, however, appeared sicker on the fourth day, when the merozoites were so numerous; and shreds of salmon-colored material, that may have been sloughed mucosa, were found in the lumen.

Levine (1942) reported that there were no lesions in light infections. The lesions described for moderate infections were very similar to those described above except for short, transverse, red streaks in the mucosa, which we did not observe. None of these chickens showed the lesions of severe infection which Levine stated represented extensive coagulation necrosis, sloughed mucosa that takes on the appearance of cottage cheese, and a caseous core in the tubular portion of the caeca.

Comparison with other Eimeriae in Chickens

There follows a discussion of the tissue stages of species of Eimeria in chickens in relation to E. brunetti, with special emphasis being placed on morphological differences that would distinguish E. brunetti from the other species found in natural infections.

Eimeria tenella develops primarily in the dilated portion of the caeca, but may be found in the lower small intestine or rectum. The generation I schizonts are found in the epithelium of the caecal glands, and the generation II schizonts, which are even larger than the generation I schizonts, are subepithelial with large merozoites. There is nothing unusual about the sexual stages which develop in the epithelium below the nuclei. The asexual stages of E. tenella could not be mistaken for those of E. brunetti which develop in the epithelium of the villi, and do not form colonies. Also the latter's generation I and II schizonts are smaller.

Eimeria necatrix has a development very similar to E. tenella except that the asexual stages occur in the small intestine, and the sexual stages in the caecum. It also develops in the gland epithelium, and forms colonies.

Eimeria acervulina can be separated from E. brunetti in tissues by the fact that it develops superficially in the epithelium above the cell nuclei. It is found in the upper small intestine and occasionally scattered in the lower small intestine, rarely in the tubular portion of the caeca. Whitish or gray areas are produced on the surface of the mucosa by swarms of oocysts. It occasionally enters the glandular epithelium. Only

one generation of schizonts was observed by Tyzzer (1929). They had 16-32 meroxoites, and appeared on the third day.

Eimeria praecox develops in the upper third of the small intestine and seems to be restricted to that area. It is confined to the epithelial cells of the villi, where a large proportion develops below the nuclei and is never found in the glands. It does not form colonies like E. tenella and E. necatrix. Generation I schizonts are similar to E. brunetti in that they develop along the sides of the villi. When mature, however, they are not as large as E. brunetti generation I schizonts, and thus can be distinguished. Other stages of E. brunetti were not found in the upper small intestine.

Eimeria mitis develops throughout the small intestine and in the tubular portion of the caeca, but is most concentrated in the upper half of the small intestine. Tyzzer (1929) found only one generation of schizonts with 6 to 24 merozoites. Forms are found below the nuclei of the epithelium of the villi and, occasionally, in the glands. Oocysts are passed on the fifth day, but their size, 16.2 by 15.5 microns, is much smaller than E. brunetti.

The developmental stages of Eimeria hagani and E. brunetti cannot be compared since the endogenous stages of E. hagani are unknown.

Eimeria maxima develops throughout the entire length of the small intestine, but mostly in the middle. It produces small schizonts in small numbers that are superficial to the nucleus. The sexual stage is the pathogenic one in this species, rather than the asexual one. The large macrogametocytes and microgametocytes develop deep in the epithelium. Tyzzer (1929) stated that the microgametocytes had a characteristic multicentric arrangement and were larger than macrogametocytes or oocysts. Since this condition had been observed in no other species, he concluded that E. maxima was distinguishable on this feature alone. In the present work, however, it has been shown that E. brunetti microgametocytes also have a multicentric arrangement and are larger than macrogametocytes or oocysts. Therefore, it can no longer be considered a distinguishing characteristic for E. maxima. The asexual stages, size, and prepatent period are the distinguishing characteristics.

SUMMARY AND CONCLUSIONS

1. Chickens were infected with a pure culture of Eimeria brunetti oocysts descended from single cell isolations in order to study the developmental stages. Tissues were taken from the upper, middle, and lower small intestine, caeca, rectum, cloaca, and bursa cloaca at approximately twelve-hour intervals and stained with Giemsa and iron-hematoxylin.

2. The excystation of oocysts was studied, and it was found that sporozoites were liberated in the intestine. They were found in the tissues of the upper, middle, and lower small intestine and rectum, and in the lumen of the above portions of intestine, as well as the caeca, at three hours. Some oocysts passed through the intestine unhatched.

3. Large generation I schizonts, measuring an average of 30 by 20 microns, and containing approximately 200 merozoites, were observed along the sides of the villi of the upper, middle, and lower small intestine at 51, 60, and 76 hours.

4. Generation II schizonts were smaller, measuring an average of 29.9 by 16.2 microns, and contained 50 to 60 merozoites. They were observed in the epithelium of the villi, primarily at the tips, on the fourth day after infection (95 hours).

5. A third type of small schizont, measuring an average of 9.8 by 8.8 microns, was observed on the fourth day (95 hours). Its significance was not determined.

6. Microgametocytes had a multicentric appearance and were larger than macrogametocytes.

7. Macrogametocytes, measuring an average of 25.2 by 22.2 microns and containing plastic granules, were observed in the lower small intestine, caeca, rectum, and cloaca lying on the basement membrane.

8. The pathology of E. brunetti was discussed. It consisted of red pin-point lesions most numerous in the lower small intestine close to the caeca, and in the narrow tubular portion of the caeca. These first appeared at 82 hours, and continued until the sixth day after infection. Shreds of salmon-colored material in the intestine and a caecal plug were observed on the fourth day after infection (106 hours). The villi of the intestine were denuded of epithelium, probably mostly by the asexual stages. Fluid, blood-tinged feces were discharged.

9. The relation of E. brunetti to the other seven known species of Eimeria in the chicken is discussed.

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PLATE I

Developmental stages of Eimeria brunetti, x 2400

Fig. 1. Sporulated oocyst of E. brunetti. (Drawn from wet mount).

Fig. 2. Empty sporocyst. (Drawn from smear)

Fig. 3. Sporozoite showing a nucleus between two refractile globules. (Drawn from smear)

Fig. 4. Sporozoite showing only one refractile globule (nucleus was not visible). (Drawn from smear)

Fig. 5. Multinucleate generation I schizont showing centrally located refractile ("eosinophilic") globule. (Drawn from section)

Fig. 6. Generation I schizont containing many developing merozoites. (Drawn from section)

Fig. 7. Generation I merozoite drawn from a tissue section showing a nucleus with a karyosome.

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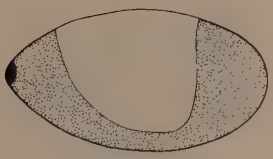

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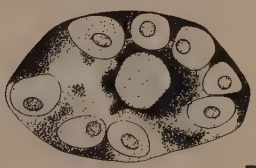

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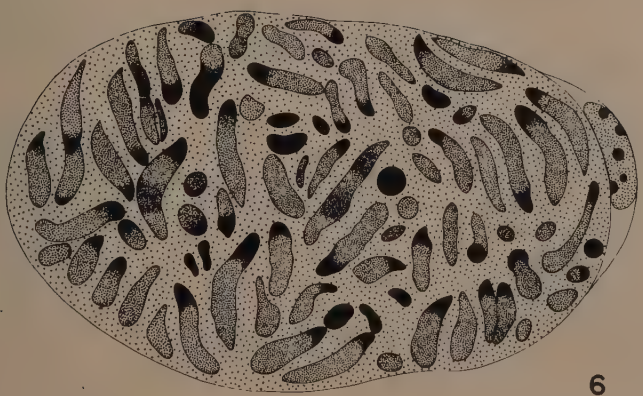

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PLATE II

Developmental stages of Eimeria brunetti, x 2400

Fig. 8. Generation I merozoite after entering a cell. (Drawn from section)

Fig. 9. Young generation II schizont with two nuclei. (Drawn from section)

Fig. 10. Small typs of schizont with approximately 12 to 14 merozoites found on the fourth day (95 hours). (Drawn from smear)

Fig. 11. Large generation II schizont. A few karyosomes in the nuclei of the merozoites are visible. (Drawn from smear)

Fig. 12. Generation II merozoite drawn from a smear.

Fig. 13. Young developing macrogametocyte showing a relatively large nucleus with a large karyosome, surrounded by a spongy-appearing cytoplasm. (Drawn from section)

Fig. 14. Mature macrogametocyte showing an ellipsoidal nucleus with a large karyosome, and a spongy-appearing cytoplasm with peripheral layer of plastic (or "eosinophilic") granules. (Drawn from section)

Fig. 15. A developing oocyst showing the outer wall formed from the plastic granules, the spongy, granular cytoplasm with faint plastic globules, the nucleus with a karyosome diminished in size, and concentration of granules around the nucleus. (Drawn from section)

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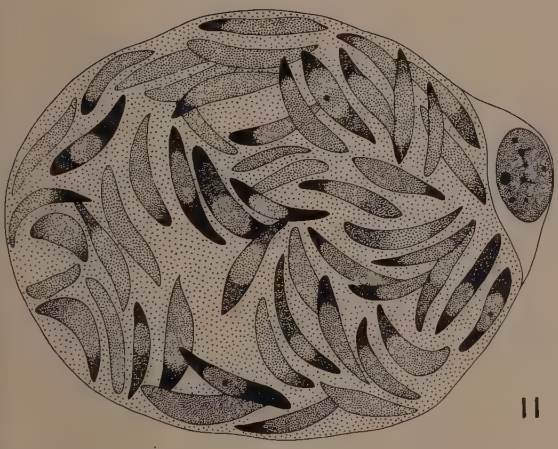

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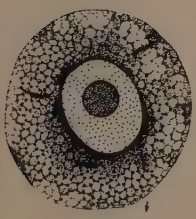

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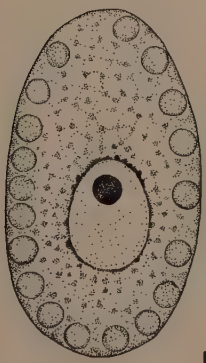

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PLATE III

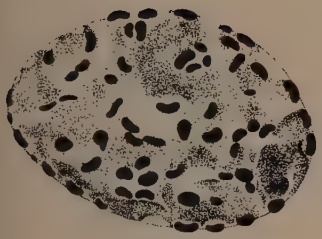
Developmental stages of Eimeria brunetti, x 2400

Fig. 16. Developing microgametocyte with lines of cleavage and dark aggregates of chromatin which later form microgametes. (Drawn from section)

Fig. 17. Developing microgametocyte with U-shaped microgametes, and cleavage. (Drawn from section)

Fig. 18. Microgametocyte with comma-like microgametes. (Drawn from smear)

Fig. 19. Microgametocyte with mature microgametes developing in whorls around residual matter. (Drawn from smear)



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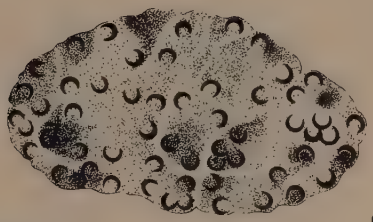

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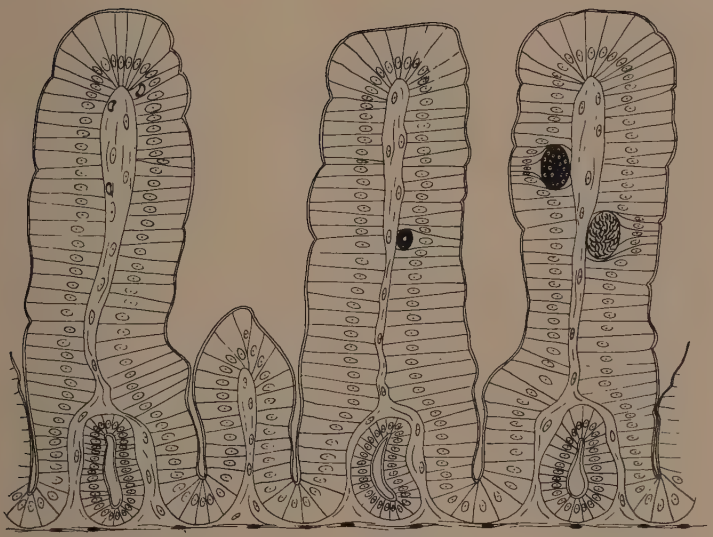
PLATE IV

Schematic Composite Drawing

Fig. 20. A schematic drawing showing the position of sporozoites and generation I schizonts in the villi of the upper small intestine. At three hours sporozoites are observed in the epithelium and the center of the villus. At 51 hours these have developed into multinucleated generation I schizonts with a central refractile globule, one of which is shown located along the side of the villus. At 60 hours multinucleated generation I schizonts are still observed along the side of the villus; also generation I schizonts with merozoites.

Fig. 21. A schematic drawing showing the position of parasites in the villi of the middle and lower small intestine. At 51 hours the parasites in the lower small intestine differ from those found in the upper small intestine, as shown in Fig. 20, in that in addition to the multinucleated generation I schizonts, and the mature generation I schizonts with merozoites, there are merozoites that have just entered cells. At 60 hours the merozoites that had entered the cells have started to develop into generation II schizonts. Some multinucleated generation I schizonts are still observed. At 76 hours multinucleated generation I schizonts and generation I schizonts with merozoites are observed in the middle small intestine. No parasites were observed in the lower small intestine at this time.



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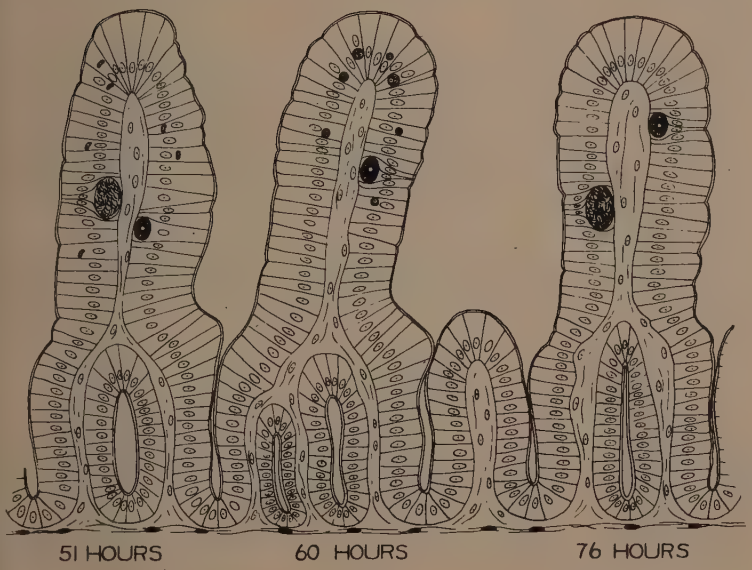

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PLATE V

Schematic Composite Drawing

Fig. 22. A schematic drawing showing the position of parasites in the lower small intestine. At 82 hours, merozoites that have just entered cells and multinucleated generation II schizonts are observed, usually below the nucleus. At 95 hours, multinucleated generation II schizonts, mature generation II schizonts, and small sized schizonts with merozoites are observed. At 106 hours merozoites that have just entered cells, young macrogametocytes, and a few multinucleated generation II schizonts are observed. During all these hours, there is some sloughing of the epithelium.

Fig. 23. A schematic drawing showing the position of parasites in the lower small intestine. At 120 hours, mature and young macrogametocytes, microgametocytes, and developing oocysts are observed. At 147 hours, all the sexual forms present at 120 hours are observed, and there is a denudation of the epithelium which leaves only the basement membrane separating the tunica from the lumen.

Fig. 24. The distribution of E. brunetti in the intestine. Circles indicate the first generation, and the black dots indicate the rest of the development.

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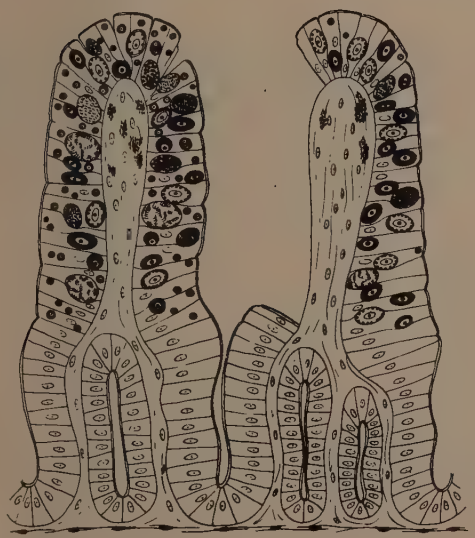

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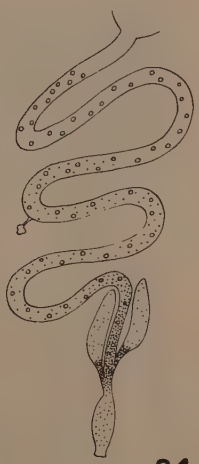

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PLATE VI

Photomicrographs

Fig. 25. The tip of a villus of the rectum (95 hours) showing large developing generation II schizonts resting on the basement membrane, numerous mature schizonts with merozoites, the damage done to the epithelium, and hyperemia. x 310.

Fig. 26. The dilated portion of a caecum (106 hours) showing the damage done by a caecal plug. A homogeneous colloidal discharge, which stained pink with Giemsa, is visible in the glands, and the same discharge charged with numerous bacteria is visible surrounding the villi. The lumen contains dried fecal matter and blood. x 125.

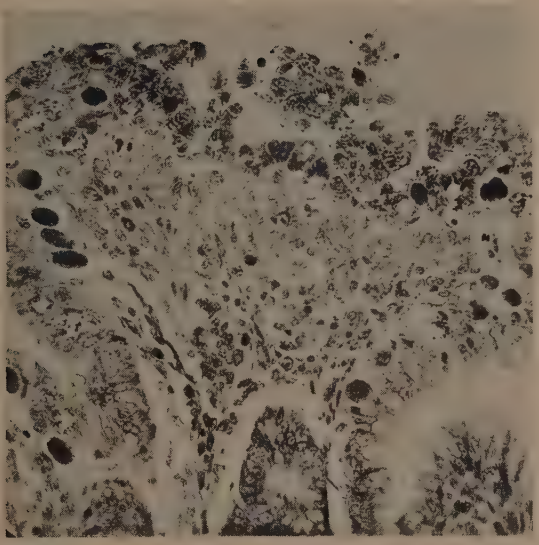

Fig. 25.

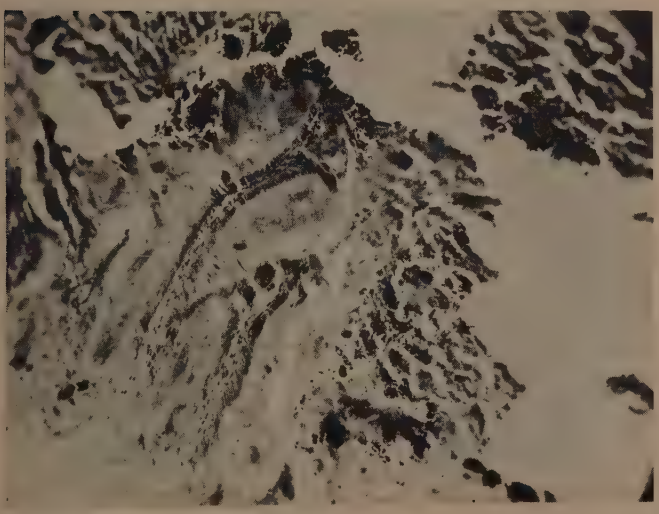

Fig. 26.

PLATE VII

Photomicrographs

Fig. 27. The tip of a lower small intestine villus (120 hours) showing a large developing microgametocyte with multiple nuclei lyin next to a mature microgametocyte with whorls of microgametes. x 310.

Fig. 28. The tip of a lower small intestine villus (120 hours) showing mature microgametocyte with microgametes and cleavage (ce ter). Also macrogametocytes, young microgametocytes, an merozoites that have just entered cells and rounded up. x 310

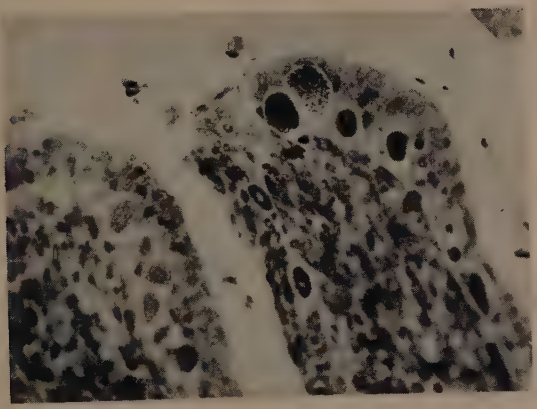

Fig. 27.

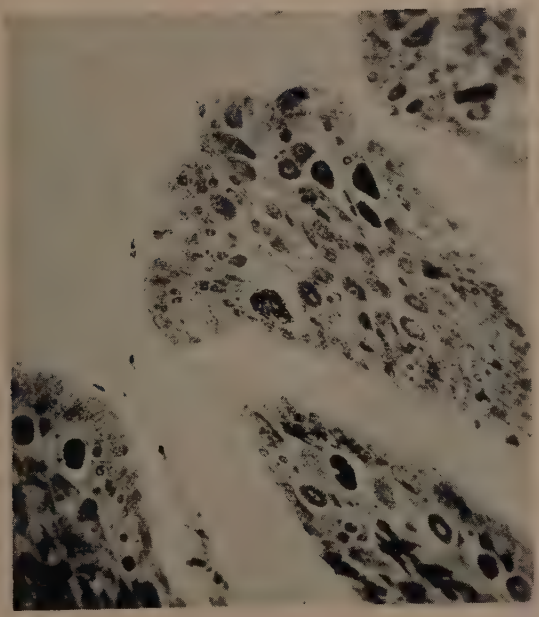

Fig. 28.

PLATE VIII

Photomicrographs

Fig. 29. The narrow tubular portion ("proximal") of a caecum (120 hours) showing many young macrogametocytes, some mature macrogametocytes, and hyperemia. x 310.

Fig. 30. A late developing oocyst with a faintly visible wall, developing microgametocytes with lines of cleavage, and a macrogametocyte. x 310.

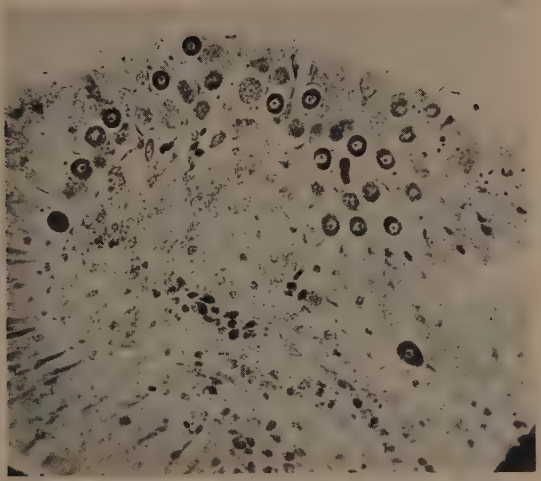

Fig. 29.

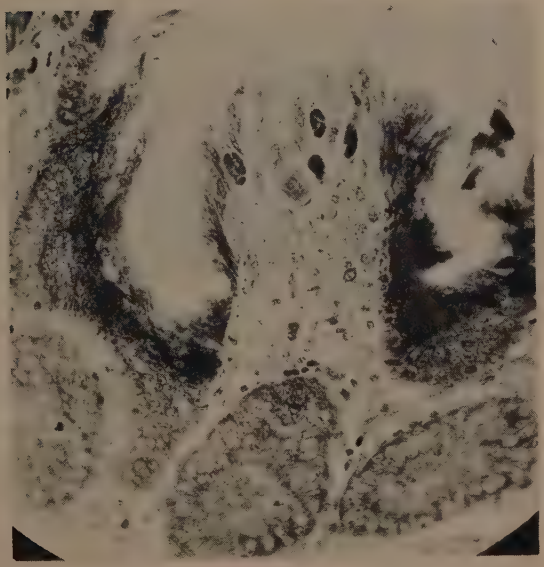

Fig. 30.

PLATE IX

Photomicrograph

Fig. 31. An area of the lower small intestine (147 hours) showing the denudation of the epithelium with only the basement membrane separating the tunica propria from the lumen, and hyperemia. x 164.

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PHYSIOLOGY OF ENDOCONIDIOPHORA FAGACEARUM BRETZ, I. FACTORS INFLUENCING GROWTH AND TOXIN PRODUCTION.[1]

P. F. Hoffman[2]

INTRODUCTION

The causal agent of oak wilt, Endoconidiophora fagacearum Bretz, produces a toxic substance in culture which, if produced in the host, may be responsible, at least in part, for symptom expression (19). As part of the search for a chemical to control the disease, some of the factors influencing growth and toxin production in liquid culture were determined.

METHODS AND MATERIALS

Most of the growth studies reported here were of cultures growing on liquid media. Measurements of growth were based on dry weight of the fungus, obtained by filtering the cultures under vacuum, washing with distilled water, drying the mats to constant weight, and weighing to the nearest mg. Agitation of cultures was obtained with a "wrist-action" and a rotary "swirl" shaker. Flasks of 125 ml. or 250 ml. capacity containing 25 or 50 mls. of medium were used unless otherwise indicated. The media were inoculated with a conidial suspension of 500,000 to 1,000,000 spores per ml. Incubation was at 25° C. unless otherwise specified. The media used in the following tests are listed in Table 1.

Tomato cuttings were used as indicator plants for assaying the toxin produced by E. fagacearum, as suggested by other workers (3, 9). The use of small cuttings 1 to 2 inches tall facilitated use of a large number of replicates and required only a few mls. of the culture filtrate for assay purposes. Tomato cuttings were placed in small vials containing the filtrate and the degree of wilting was estimated after 24 or 48 hours. The results of toxin assay were recorded on a scale of 0 to 10 in which 0 represented a turgid healthy plant and 10 a completely wilted or necrotic plant. When toxin titer was high the lower portion of the stem quickly collapsed, preventing upward movement of the toxin and, therefore, the leaves remained turgid. This was considered maximum injury and was recorded as 10. Other methods also tested, included inhibition of bacterial growth, destruction of membrane permeability, and inhibition of protoplasmic streaming. None was considered to be superior to the tomato assay.

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[1] Journal paper No. J-2354 of the Iowa Agricultural Experiment Station, Ames, Iowa, in cooperation with the Iowa State Conservation Commission Project No. 1047. In part taken from a thesis (5) submitted to the graduate faculty of Iowa State College, in partial fulfillment of the requirements for the degree Doctor of Phylosophy. A portion of this work was carried out at the Illinois State Natural History Survey, Urbana, Illinois.

[2] Present address: Monsanto Chemical Co., St. Louis, Missouri. The writer is indebted to Dr. W.H. Bragonier for helpful advice during the course of the work and for criticism of the manuscript.

TABLE 1

Media used in growth studies with Endoconidiophora fagacearum

Constituent	Medium number					
	1	2	3	4	5	6
	Concentration in grams per liter					
NH_4NO_3	0.5	1.0	0.5		1.0	
Yeast extract	0.5	4.0	0.5		2.0	
l-asparagine					1.0	1.0
KH_2PO_4	0.5	1.0		0.5	1.0	0.5
$MgSO_4 \cdot 7H_2O$	0.5	0.5	0.25	0.25	0.5	0.25
d-glucose	15	15	10	10	25	5
	Concentration in milligrams per liter					
$ZnCl_2$				.54		.54
$MnSO_4 \cdot 4H_2O$				.14		.14
$(NH_4)_6MO_7O_{24} \cdot H_2O$				.03		.03
$CuSO_4$				.14		.14
$FeSO_4$				.20		.20
$FeCl_3$		1.0			1.0	

MacIlvaine's citrate-phosphate buffer was used in pH studies for the maintenance of constant substrate pH. This buffer was diluted one-tenth to maintain a low concentration of salts in the medium. Approximate determinations of pH were made with p-Hydrion paper. For greater accuracy a Beckman pH meter was used.

RESULTS

Agitated and quiet cultures. Growth of E. fagacearum in quiet and agitated cultures (Fig. 1) reached the same peak on medium 1, however, growth in quiet culture lagged behind agitated culture. Toxin production was similar in both agitated and quiet cultures. Acidity of the medium increased more rapidly in agitated than in quiet culturing because of the faster initial growth rate. The toxic substance was detected by the sixth day in both cultures, at which time pH of the medium was falling rapidly. A toxin index of 10 was obtained from the eighth to the sixteenth day at which time the experiment was concluded. The failure of E. fagacearum to show a more pronounced increase in growth in response to agitation is not typical of most fungi (4). In the "lean" media used in these studies, media having a low concentration of sugar and salts, however, growth was largely subsurface with mat formation slow in developing.

Carbon source. E. fagacearum grew better on a basal medium supplemented with potato-starch than when supplemented with any one of six other carbon sources tested in one experiment (Table 2). Toxin activity, however, was greatest when the basal medium was supplemented with d-glucose, maltose, d-fructose, or sucrose. Toxin activity in the potato-

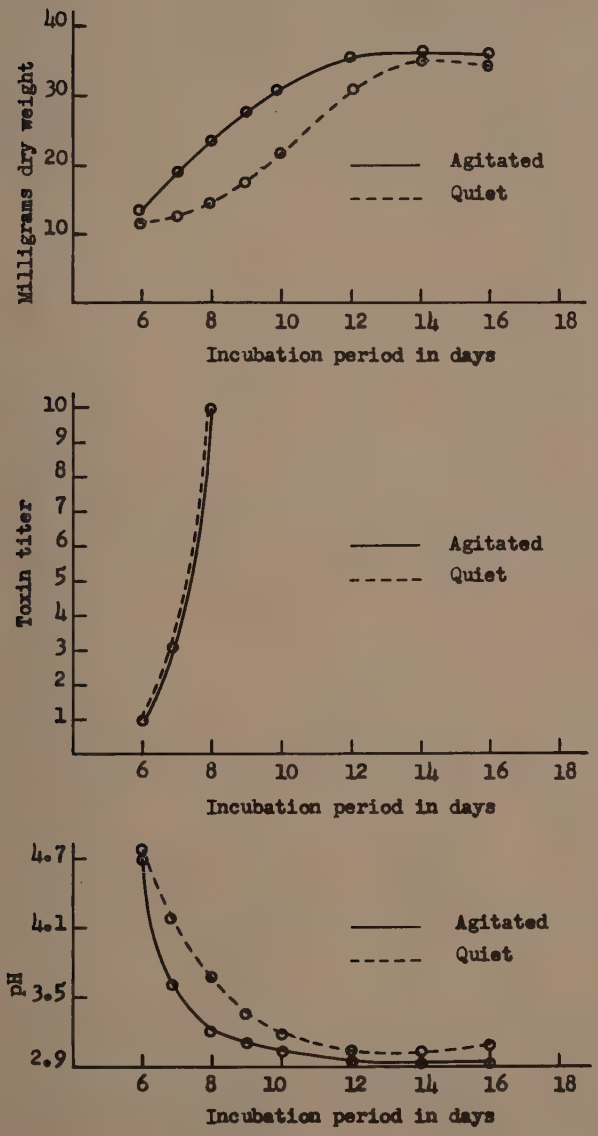

Fig. 1. Growth and toxin production by Endoconidiophora fagacearum in basal medium 1 and the change in pH of the medium in agitated and quiet culture.

TABLE 2

Growth and toxin production of E. fagacearum on medium 1 supplemented with various carbon sources

Carbon source	Average growth mg dry wt.			pH			Toxin titer*		
	20 days	30 days	38 days	20 days	30 days	38 days	20 days	30 days	38 days
d-Glucose	72	69	68	2.78	2.98	2.94	10	10	10
Maltose	112	117	131	2.91	2.95	3.02	10	10	10
d-Fructose	133	162	160	3.08	3.28	3.48	10	10	10
Sucrose	99	104	137	3.16	2.81	2.97	10	10	10
Lactose	4	13	16	7.10	7.77	7.53	2	2	0
Potato starch	189	233	234	3.63	4.57	4.48	10	3	2
d-Mannitol	37	21	31	6.55	7.28	6.62	4	3	0

* Based on a scale of 0 to 10 where 10 is a completely wilted tomato cutting and 0 is a normal one.

starch medium dropped presumably because of the decrease in acidity. In another test of 25 carbon sources, dextrin and raffinose supported the most growth. Sucrose, soluble starch, fructose, glucose, maltose, and inulin were almost as good (Table 3).

TABLE 3

Growth of E. fagacearum after 18 days on medium 5 supplemented with 25 carbon sources.

Carbon Source	Growth mg. dry wt.	Carbon Source	Growth mg. dry wt
Dextrin	62	l-Arabinose	19
Raffinose	56	Melezitose	17
Sucrose	43	l-Xylose	15
Soluble starch	41	Dulcitol	15
Maltose	39	Lactose	14
d-Glucose	39	Salicin	14
d-Fructose	39	Sodium succinate	14
Inulin	37	Sodium acetate	7
d-Mannose	33	Rhamnose	5
d-Galactose	30	Ammonium oxalate	2
d-Sorbitol	23	Sodium citrate	0
Cerelose	23	Calcium malate	0
d-Mannitol	19		

Incubation period. Toxin production by the fungus in basal medium 1 was sufficient to produce incipient wilting of tomato cuttings from about the sixth to the eighth day. At the end of 26 days filtrates contained enough toxin to cause incipient wilting of cuttings when diluted to 6.25 per cent, (Fig. 2). Since assay by dilution of filtrates required a large number of plants, particularly when there were many treatments, the method was abandoned in favor of one in which wilting percentages were estimated for individual plants.

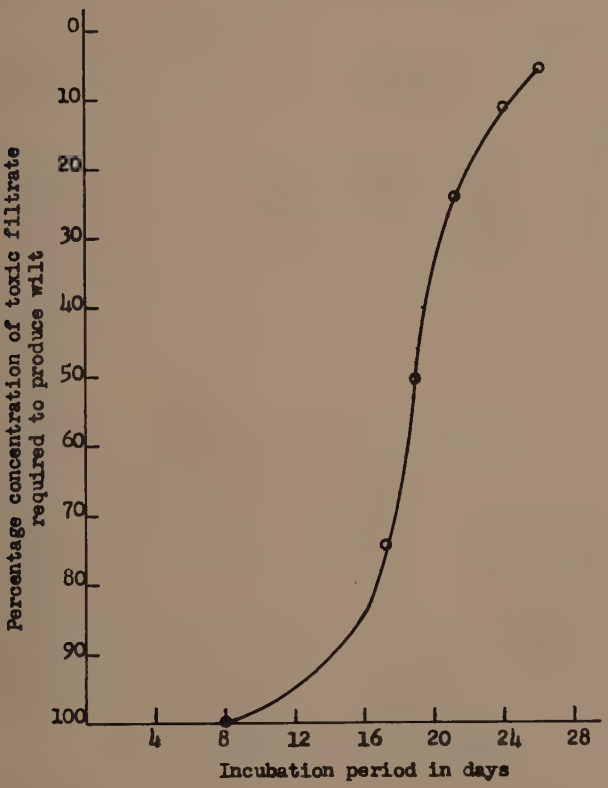

Fig. 2. The influence of incubation period on toxin production by E. fagacearum in basal medium 1 at 25°C.

Hydrogen ion concentration. To determine optimum pH for growth in liquid culture the fungus was grown in medium 2 adjusted to a range of pH values with 0.1N HCl or 0.1N NaOH, for 19 and 30 days. Substrate pH could not be maintained at a constant level without buffering; therefore MacIlvaines citrate-phosphate buffer was used. This buffer diluted one-tenth maintained a relatively constant pH over a range of about 3.5 to 7.0 except for a slight initial drop (Fig. 3).

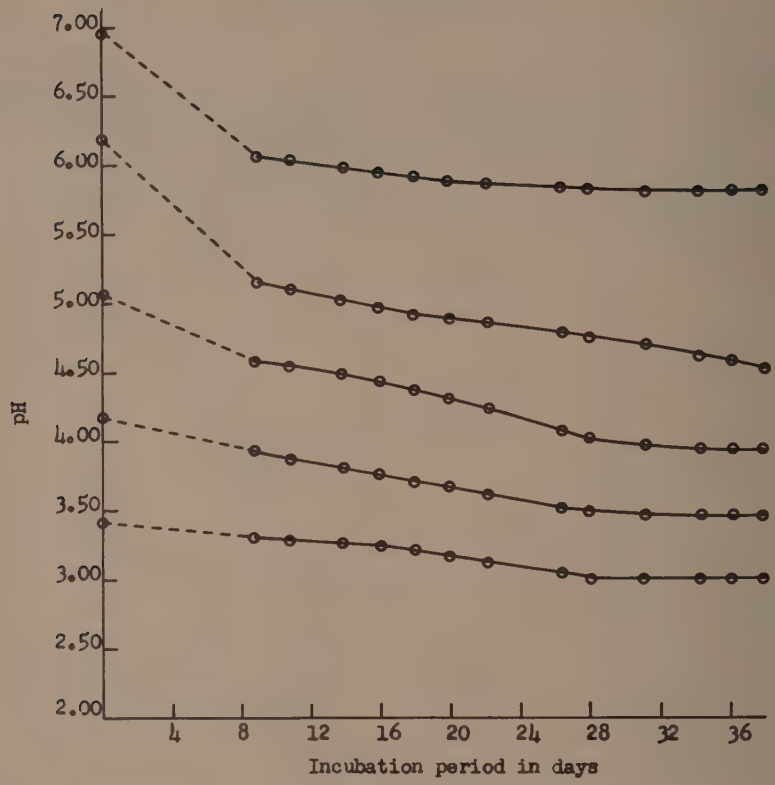

Fig. 3. Effectiveness of MacIlvaine's citrate buffer, in maintaining the pH of medium 3 in which E. fagacearum was growing.

Maximum growth was obtained in media originally adjusted to pH 4.. and 5.1 (Fig. 4a) in 20 days. In contrast, maximum toxin activity wa obtained by the fourteenth day in media adjusted to pH 3.4 and 4.2 (Fig 4b). Toxin production or activity appeared therefore to be somewhat in dependent of growth and was favored by a lower pH. The optimum pH fo growth of E. fagacearum has been reported as 5.0 to 7.0 on a solid sub strate (9) and 4 to 5 (2), 4 (8) or 6 to 7 (1) in liquid media.

Temperature. The optimum temperature range for growth of the fun gus on agar plates has been determined as 20° to 28°C. by Young (9) Liquid cultures sampled at intervals from the sixth to the thirty-sixth da indicated that it was approximately 20°C. (Fig. 5). At the end of 36 day total dry weight on basal medium 1 was still increasing at 10° and 30°C. while autolysis was occurring at 15°, 20° and 25°C.

Nitrogen source. Asparagine was superior to ammonium nitrate in a experiment to test the influence of two nitrogen sources on growth an toxin production of the fungus (Fig. 6). No difference was found in th amount of toxin produced in either medium. Enough toxin to produc maximum wilting was obtained by the fourteenth day in both media. A this time pH of the filtrates had dropped to approximately 3.0.

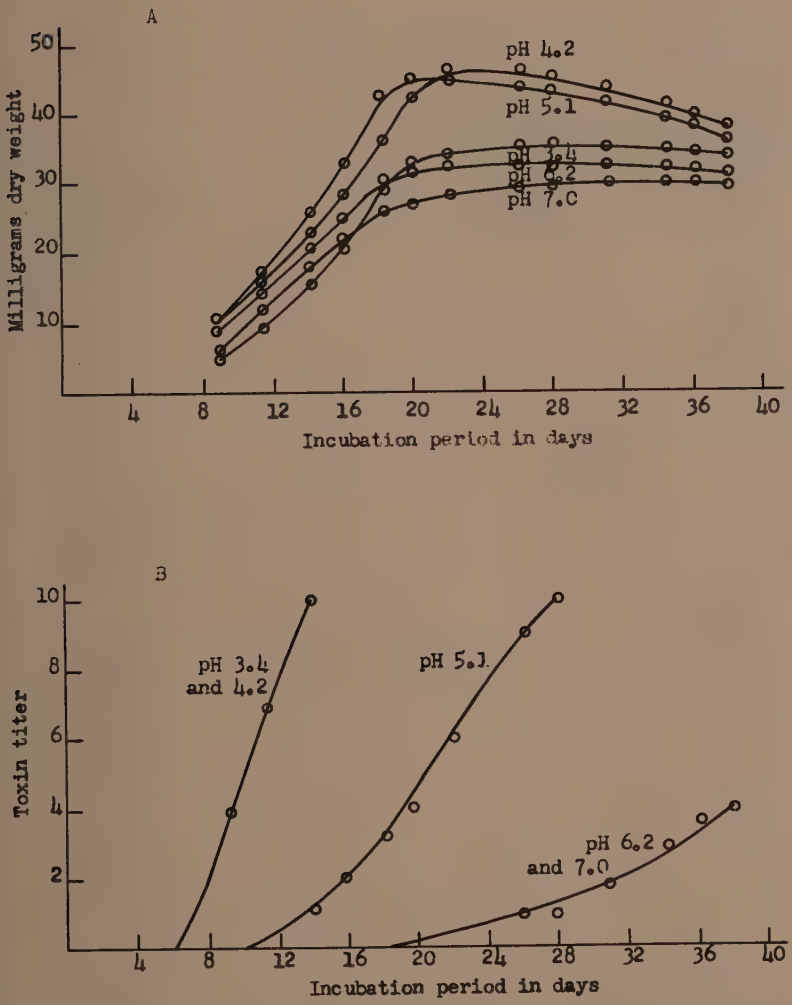

Fig. 4. Effect of time of incubation and pH of the medium on growth (A) and toxin activity (B) of E. fagacearum in MacIlvaine's citrate buffer, medium 3.

It was found that l-asparagine was superior to 24 other nitrogen sources for growth when cultures were incubated for 33 days. After 10 days growth no difference could be detected between media supplemented with l-arginine hydrochloride or l-asparagine (Table 4). Substitution of peptone or yeast extract for single sources was effective at ten days but not after 33 days.

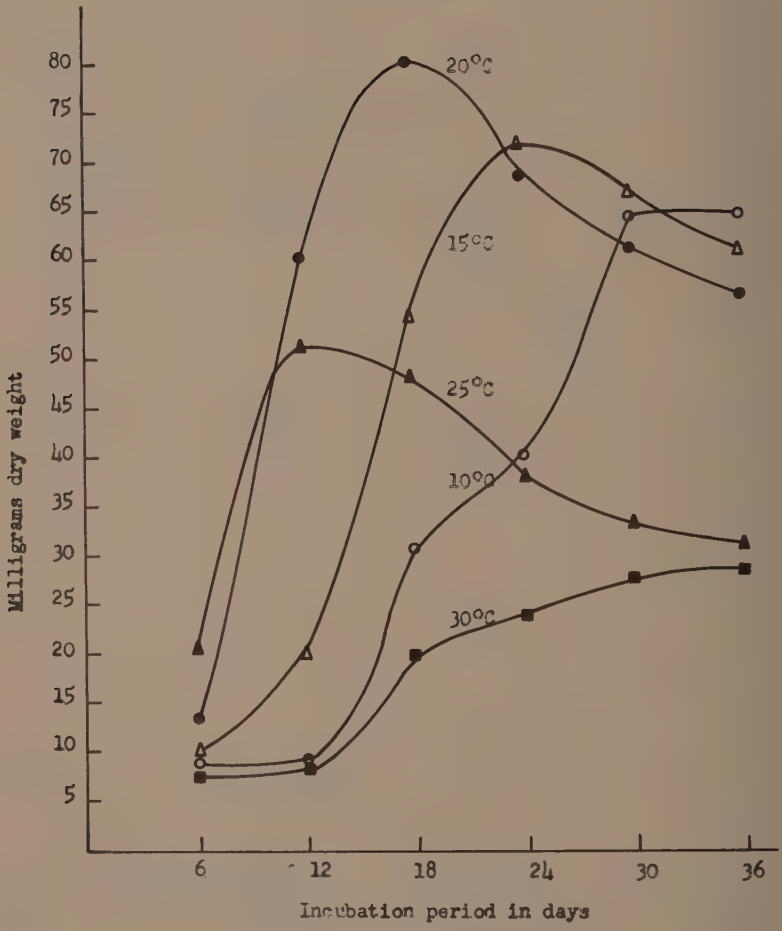

Fig. 5. Effect of temperature on growth of E. fagacearum in basal medium 1.

Vitamin requirements. Some fungi lack the ability to synthesize certain vitamins or vitamin moieties (7). Four of these vitamins, thiamin, biotin, i-inositol, and pyridoxine, were added to purified (6) basal medium 6, alone and in combination to determine if E. fagacearum was able to synthesize these vitamins. Of six isolates of the fungus that were tested, growth of one isolate, F3, was stimulated somewhat by the addition of thiamin, and growth of another isolate, W4, by the addition of thiamin and biotin. The other four made satisfactory growth on media lacking these four vitamins (Table 5).

Source of isolates. Nine isolates were examined for differences in toxin production. These isolates had been obtained from two states and from three species of oak. Although they had been in culture for periods

TABLE 4

Growth of E. fagacearum in medium 4 supplemented with different nitrogen sources

Nitrogen source	Average growth, mg. dry weight	
	Run 1 (10 days)	Run 2 (33 days)
l-asparagine	33	231
Yeast extract	36	200
l-arginine HCl	35	198
l-glutamine		193
l-glutamic acid	13	147
Peptone	35	104
Urea		91
Citrulline		58
dl-aspartic acid	10	50
$NH_4H_2PO_4$	14	46
Ornithine		30
dl-alanine	12	
l-cystine	10	
dl-tryptophane	8	
Glycine	8	
$NaNO_3$	7	
dl-serine	6	
dl-methionine	6	
dl-threonine	6	
dl-leucine	6	
dl-phenylalanine	5	
l-lysine HCl	5	8
dl-histidine	5	8
dl-isoleucine	4	
l-cysteine HCl	2	4
No nitrogen	1	3

TABLE 5

Growth of E. fagacearum on purified Medium 6 Supplemented with four Vitamins

Vitamins	Concentration in micrograms/l	Growth in mg of dry weight					
		Isolate					
		1	EMSt-1	F-3	W-4	PP332	MP312
Thiamin HCl	100	28	32	38	30	26	46
Biotin	5	24	34	24	26	30	42
Thiamin HCl and Biotin	100 + 5	30	28	36	42	32	45
i-Inositol	5000				20	22	40
Pyridoxine HCl	100				18	24	38
No vitamins		26	30	22	22	32	41

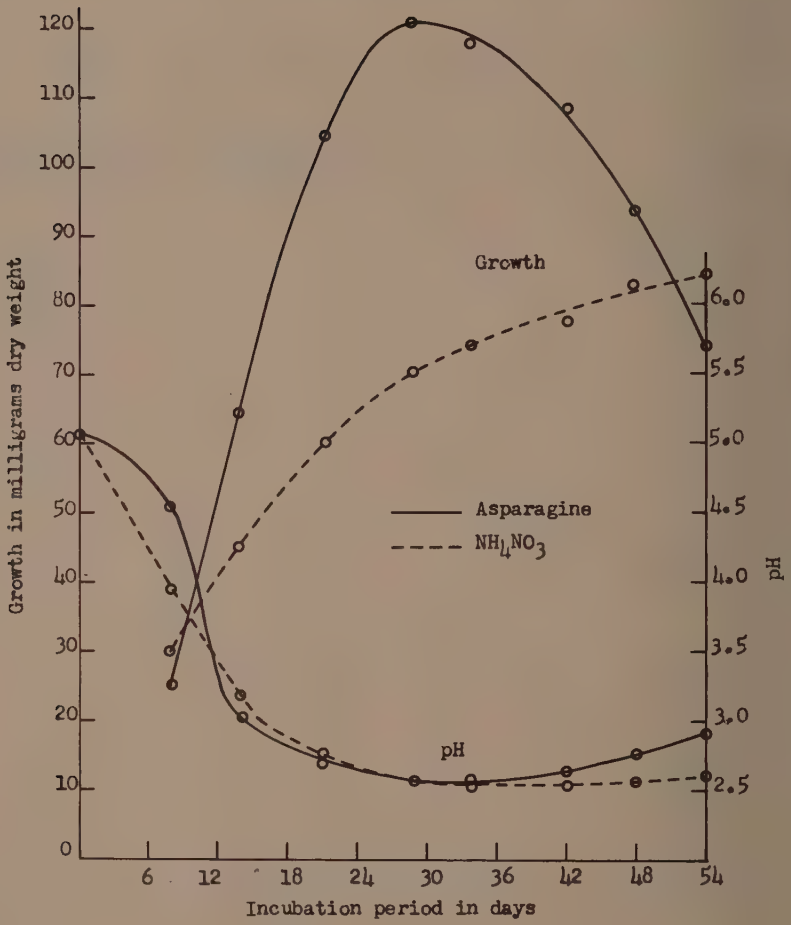

Fig. 6. Growth of E. fagacearum on two nitrogen sources in medium 4

of one to four years, they were still pathogenic to red oak seedlings an able to produce toxin. They were grown in medium 5 in both agitated an quiet culture for 26 days. All cultures grew well and, when assayed b the tomato cutting method, were found to have produced sufficient toxin t give the maximum reaction (Table 6).

Natural media. Growth of the fungus was abundant on extracts obtaine from oak wood and on chips, sawdust, and twigs of red and bur oak. Afte autoclaving sawdust with a small quantity of water, pH of the medium wa approximately 4.8. Oak wood extracts as substrates for the fungus i toxin tests were unsatisfactory because tomato cuttings wilted in uninocu lated media.

TABLE 6

Growth and toxin production of several isolates after twenty-six days in agitated and quiet culture in medium 5

Isolate	Source	Year Isolated	Average growth - mg. dry weight		Toxin titer*		Check
			Quiet	Agitated	Quiet	Agitated	
EM R150	Q. borealis	1950	148	107	10	10	0
MP 312	"	1947		140		10	0
PP 332	"	1948	125	86	10	10	0
AR 150	"	1950	132	96	10	10	0
AC 1R	"	1949	126	119	10	10	0
PK 1 H3	Q. ellipsoidalis	1949	119	106	10	10	0
PKR 50	"	1950	151	100	10	10	0
PKR 150	"	1950	141	86	10	10	0
Ind St 2-2	Q. velutina	1950	126	96	10	10	0

* Based on a scale of 0 to 10 where 0 is a normal tomato cutting and 10 is one completely wilted.

Toxin-pH relationship. The toxic substance active in tomato cuttings in filtrates at pH 3.0 to 4.0 became partially inactive when the filtrates were adjusted to pH 5.4 and almost completely inactive when the filtrates were adjusted to pH 8.0. On readjustment to pH 3.0 full activity was regained, indicating a reversible reaction. Uninoculated media adjusted to pH 3.0 caused no wilting, therefore wilting could not be ascribed to the acidity of the medium.

SUMMARY

Neither total growth nor toxin activity, as assayed by tomato cuttings, was stimulated by agitating cultures of E. fagacearum. In these experiments small volumes of liquid were used with a resulting large surface area to volume ratio. When the volume of medium per flask was increased or when "richer" media were used, stimulation of growth was obtained by agitation. Sufficient toxin to produce incipient wilting of tomato cuttings appeared in liquid media by the eighth day and the activity increased rapidly through the twenty-sixth day. At that time filtrates diluted to 6.25 per cent contained sufficient toxin to produce wilt of tomato cuttings.

In buffered media maximum growth was obtained in cultures originally adjusted to pH 4.2 and 5.1 but maximum toxin activity occurred in cultures adjusted to pH 3.4 and 4.2. Optimum temperature for growth in liquid culture was about 20°C.

Potato starch, dextrin, raffinose, d-glucose, maltose, d-fructose, sucrose, soluble starch and inulin supported satisfactory growth of the fungus. Asparagine was superior to ammonium nitrate as a nitrogen

source for growth. Equivalent amounts of toxin were produced on bo materials. Of 25 nitrogen sources tested, l-asparagine supported th most growth after 33 days incubation but was no better than yeast extrac l-arginine HCl, or peptone at the end of 10 days incubation.

Of six isolates of the fungus tested for their ability to synthesize fou vitamins, thiamin, biotin, i-inositol, and pyridoxine, one could synthe size only limited amounts of thiamin and another only limited amount of thiamin and biotin. Growth of the other four isolates was not stimu lated by the addition of vitamins to purified media.

Nine isolates of the fungus, all pathogenic, could not be separated c the basis of toxin production.

Water extracts of oak chips and sawdust were good substrates for fun gus growth but were unsatisfactory in toxin assays because tomato cut tings wilted in the uninoculated medium.

Toxin activity in tomato and oak cuttings was directly correlated wit increasing acidity. Below pH 4.0 a toxin index of 10 was usually obtaine

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THE WINTER-SPRING MOVEMENTS OF THE RING-NECKED PHEASANT IN NORTHERN IOWA[1]

Henry G. Weston, Jr.[2]

In the management of the ring-necked pheasant (Phasianus colchicus) n Iowa, an assessment of the value of state-owned marshes in maintaining large winter pheasant concentrations was needed. This was accomplished in part by means of intensive observations on the pheasant at Birge and Grass Lake Game Areas, Emmet County, two state-owned marshes, during the winter periods of 1948 through 1950. In the evaluation it was essential to determine the relative distance of spring dispersal of pheasants from such sites. Hence, the over-all aim of this investigation was to study the winter behavior of wild-reared pheasants, primarily their movements, and their subsequent spring dispersal from these areas. Observations on sex ratios and spring dispersal were made on the adjacent farmland in north-central Emmet County, Iowa, and the south-central portion of Martin County, Minnesota. Field work was conducted from February 14 through August 9, 1948; from January 4 through June 8, 1949; and from January 5 through May 27, 1950.

Emmet County, Iowa, and Martin County, Minnesota, are entirely within the Wisconsin glacial drift soil area. The topography is gently undulating to rolling. Ninety-eight per cent of Emmet County is in farm and (4). Corn, oats, soybeans, and hay are the chief field crops, while the raising of hogs comprises the main livestock industry.

BIRGE LAKE GAME AREA

Birge Lake Game Area (Fig. 1) lies in Emmet County, Iowa, at the corner of four sections: 12 and 13 in Ellsworth Township and 7 and 8 in Lincoln Township. This area, a state-owned public shooting ground, includes 137 acres of drained bottomland and adjacent, gently rolling, rounded hills. A county drainage ditch constructed about 30 years ago divides the area lengthwise.

At the center, 6.5 acres of bottomland, moist underfoot throughout

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The author is indebted to those who helped make this investigation possible. The leadership of Dr. T.G. Scott, U.S. Fish and Wildlife Service, was invaluable during the 1948 phase of this study. Greatest indebtedness is due Dr. E.L. Kozicky, U.S. Fish and Wildlife Service, for his guidance and help during the final two years. Thanks are also expressed to Dr. G. O. Hendrickson, Department of Zoology and Entomology, for his ready counsel and guidance and to the author's wife for her constant patience, consideration and encouragement.

Now at Grinnell College, Grinnell, Iowa.

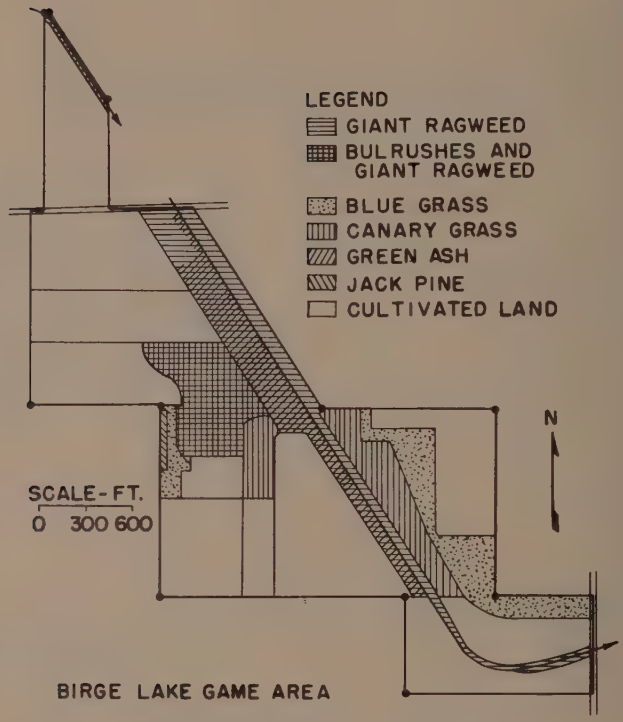

Figure 1. Distribution of dominant vegetation found on Birge Lake Game Area, 1948-1950.

much of the growing season and flooded temporarily after heavy rains, i covered with a mixture of river bulrush (Scirpus fluviatilis), dark gree bulrush (S. atrovirens), giant ragweed (Ambrosia trifida), and nettl (Urtica gracilis). A dense stand of giant ragweed, from 50 to 180 feet i width, runs parallel through the area along each side of the drainage ditch Two stands of reed canary-grass (Phalaris arundinacea) occur at th south center of the area, a 4.0 acre stand west and an 8.5 acre stan east of the drainage ditch. Kentucky bluegrass (Poa pratensis) blanket the low, rolling hills east and west of the center of the area.

Several species of trees appear on the Birge Lake Game Area. Larg eastern cottonwoods (Populus deltoides), white willows (Salix alba), peach leaf willows (S. amygdaloides), and box elders (Acer negundo) occur in termittently along the drainage ditch. Several hundred green ash (Frax inus pennsylvanica var. lanceolata), 8 to 10 feet tall, occur at the cente of the area scattered through the giant ragweed along the west ditch bank On the rise at the boundary fence west of the center, a stand of jack pin (Pinus banksiana), 8 to 10 feet tall, covers a 175 by 50 foot plot of ground Scattered black locust (Robinia pseudoacacia), 8 to 10 feet tall, appear o

the west slope near the center of the area east of the drainage ditch. In 1948, 62 per cent (85.5 acres) of Birge Lake was leased for farming, of which 44.5 acres were in permanent pasture and hay crops. The remaining acres are rotated annually (corn, soybeans, and barley).

GRASS LAKE GAME AREA

The Grass Lake Game Area (Fig. 2), a state-owned public shooting ground, was artificially drained and fenced some 30 years ago. Located in sections 16 and 17 of Ellsworth Township, Emmet County, Iowa, it includes 173 acres of bottomland. A drainage ditch running northeast-southwest through the center divides it into east and west halves.

Marsh vegetation covers 14 acres at the southeast quarter of Grass Lake and 10 acres at the east center of the area. River bulrush dominates this vegetation, although dark green bulrush, soft-stemmed bulrush (Scirpus validus), sedge (Carex rostrata), and juncus (Juncus spp.) appear intermixed at the periphery of the river bulrush. Broad-leaved cat-tail (Typha latifolia) occurs scattered throughout the marsh vegetation. During the wet periods of the year, some water gathers in these two parts of

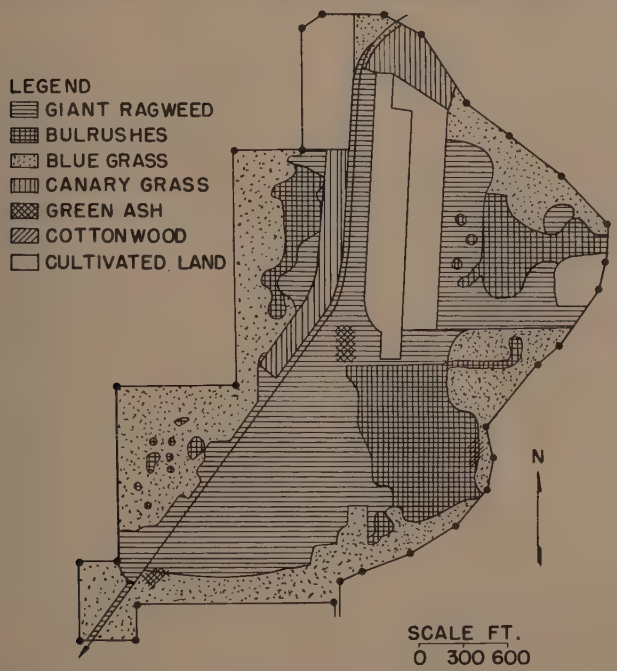

Figure 2. Distribution of dominant vegetation found on Grass Lake Game Area, 1948-1950.

the old lake bed. Small, isolated patches of marsh growth also occur west of the drainage ditch.

Fifty-two other acres are covered with a dense, pure stand of giant ragweed, except for isolated patches of nettle. Much of the periphery of Grass Lake is covered by a generally solid growth of Kentucky bluegrass. Herbaceous vegetation, including sweet clover (Melilotus alba and M. officinalis), black mustard (Brassica nigra), common dandelion (Taraxacum officinale), red-seeded dandelion (T. erythrospermum), Canada thistle (Cirsium arvense), common milkweed (Asclepias syriaca), red top (Agrostis hymenalis), wild rye (Elymus canadensis), and timothy (Phleum pratense), occurs both scattered and in isolated patches in the bluegrass.

Species of trees on Grass Lake Game Area include the eastern cottonwood, white and peachleaf willows, box elder, and green ash. Lone box elders are present along the banks of the drainage ditch, while at the center of the area, a stand of young green ash is mixed with the giant ragweed. Eight- to twelve-foot tall willows and cottonwoods occur isolated and in small clumps at the northeast and southeast corners. In 1948, 13 per cent (22.5 acres) at the north end was leased for farming. These acres are rotated annually (corn, oats, soybeans, barley, and brome hay).

Field Techniques

During the winter field activities on the Birge and Grass Lake Game Areas centered around the trapping and banding of pheasants; during the spring, on the dispersal of pheasants. Trapping of pheasants was most successful during the winter months when snow and inclement weather forced the birds to concentrate where cover and food were available. The degree of concentration depended on one, or some combination, of four factors: weather, food, vegetative shelter, and the number of birds.

Trapping and banding during the first winter period (1948) were begun February 1. Field work was initiated in the first week of January during 1949 and 1950.

All pheasants were captured by trapping, except for the night of March 12, 1948, when part of the evening was spent night-shining pheasants from a car. Pheasants roosting on the ground were blinded by a spotlight and captured with a net, banded, and released. The trap used was a modification of the Ohio pheasant trap (7). Aside from reducing the dimensions and substituting materials, no changes were made in the structural shape of the trap. Trapping bait consisted solely of field corn (Zea mays).

Most of the trapping was undertaken during the first half of the morning. In addition to early morning trapping, traps were set for late morning and afternoon catches whenever practicable.

No one trap was used more than three or four consecutive days. While one or more traps were idle, activities were shifted to other traps or from one study area to the other. Poor results at a trap were due to any one or a combination of four factors; weather, food, cover, and/or disturbance of birds in the vicinity of the traps. In regard to the food factor, the coverage of waste grain by snow, especially corn in machine-picked corn fields, was correlated with trapping success.

At a visitation, a trap containing birds was entered quickly and all birds were placed in burlap sacks; three to six birds to a sack. The birds were then taken to a car or panel-truck, where they were marked, banded and released.

Banding procedures were uniform throughout. Two types of leg bands were utilized; an aluminum, numbered, butt-end Iowa State Conservation Commission band was placed on one leg and a colored, over-lapping, plastic band was placed on the other.

As an aid to field identification, two types of tail markers were used in the 1948-49 winter. One, similar to that used by Edminster (2) in ruffed grouse, was a previously enameled feather glued to one of the center tail feathers of a trapped pheasant. This was used only in the 1948-49 winter, for it was found that the host feather with the attached colored marker pulled out quite readily.

The second type of tail marker, called a painted marker, consisted of applying two coats of paint directly on one of the center tail feathers of each bird trapped. A quick-drying auto touch-up paint ("Duplicolor") was used. A stream of warm air from an automobile heater dried each coat of paint in about 30 seconds. The same color combinations were employed for Birge and Grass Lake Game Areas as were utilized on the previously enameled feathers. Between January 13 and 26, 1949, this method of painting one of the tail feathers was used on 32 birds. The method proved time consuming and necessitated excessive handling of the birds.

Beginning January 26, 1949, a modification of the painted marker was used (6). First, a coat of DuPont Duco household cement was applied to a tail feather. The cement dried quickly and provided an excellent base for a fast-drying, colored lacquer. The outer inch or two of each feather painted was clipped off as a precaution against freezing to the ground and as an additional means of field identification. The change to Duco auto lacquer was made after the "Duplicolor" was found to fade.

A total of 790 pheasants was marked during the two years that painted tail markers were utilized. Eight feathers (one per cent) were found: five in retrapping operations and three in subsequent field studies. In contrast to this method, 43 enameled markers (14 per cent) of 303 marked were found: 33 in retrapping and 10 in subsequent field work.

More definite conclusions as to the efficiency of the painted tail markers were drawn from data gathered at the Iowa State Conservation Commission Game Farm near Boone, Iowa. On October 29, 1949, with the assistance of Mr. Clyde Updegraff, the tails of 20 pheasants were marked by means of the painted tail method. The pheasants were enclosed in an eight-acre pen with several hundred other pheasants. A check of 16 of the birds four months later disclosed that five (25 per cent) still had markers in good shape; eight (40 per cent) showed the outer edges of the web of the rectrices to be slightly pecked; three (15 per cent), although badly pecked, were readily distinguishable with the bird in hand. No markers were lost. These data indicated that the painted tail markers were more efficient than the enameled type markers.

No known loss of the aluminum butt-end bands was noted. Two females, banded in March 1948, were retrapped in February 1949, and the aluminum leg band on each was in good condition. One of these two returns still had her plastic band which, though thin, was in good condition. In

January 1950, two other females, banded in 1948, were retrapped at the Grass Lake Game Area. Each had a metal band in good condition. One still had a plastic band.

Two methods were used in the field during the winter to determine number and sex of birds. On each winter concentration area, flushed birds were counted while traversing the area on foot. On the 39 sections surrounding the two study areas, an index to the number of birds was determined by direct observation from a car. Records were kept on the number and location of pheasants observed and whether any were marked.

Methods utilized for obtaining data on winter cover relationships fell into two main categories: (1) identification and mapping of cover type components, and (2) repeated observations of pheasants and their sign as related to the cover present. Data on winter field behavior were gathered mainly by field observation. Climatological data relating to temperature, precipitation, barometric pressure, and wind velocity were also collected.

During the spring phase of the investigation, data were gathered on the location of birds and on presence or absence of markers by driving the roads surrounding these areas, and searching for marked birds, with the aid of an eight-power binocular. An estimate was also maintained on the number of pheasants remaining on the two areas.

Winter Behavior

Winter weather. The 1947-48 winter was characterized by a general absence of snow which undoubtedly was advantageous for the survival of pheasants, whereas much of the 1948-49 winter recorded near zero temperatures (F.) with at least two inches of snow present on the ground during most of January and February. Throughout much of the 1949-50 winter period, 5 to 10°F. below normal temperatures were recorded, and scattered light snowfalls occurred.

Winter censuses. During this investigation, a marked year to year increase in the maximum number of pheasants wintering on the Grass Lake Game Area (Fig. 3) and on 39 sections of surrounding land was apparent. The Birge Lake Game Area did not parallel this steady yearly increase; in 1950, a decrease in the winter pheasant population was noted (Fig. 4). Little annual change was noted in the number of birds remaining to nest on the two areas.

Each winter an approximation of the maximum pheasant numbers at each area was determined. Fluctuations in numbers were correlated with weather (Table 1). Snow depth appeared to be the more predominant among the weather factors influencing the degree of concentration. Snow ground cover of about five inches or more concealed the food supply. Consequently, pheasants tended to concentrate on the study areas where standing field corn and feeding stations were maintained and where an abundance of natural vegetative cover was available.

While driving roads in the winter of 1949, 556 pheasants at 17 different locations were observed on 39 sections of land surrounding Birge and Grass Lake Game Areas. These 17 concentrations occurred in the vegetative cover at 11 farm shelterbelts, three drainage ditches, one roadside stand of trees, one railroad right-of-way, and one field of corn grazed by sheep. In 1950, 673 birds were found at 14 farm shelterbelts, three drainage ditches, and three roadside stands of trees.

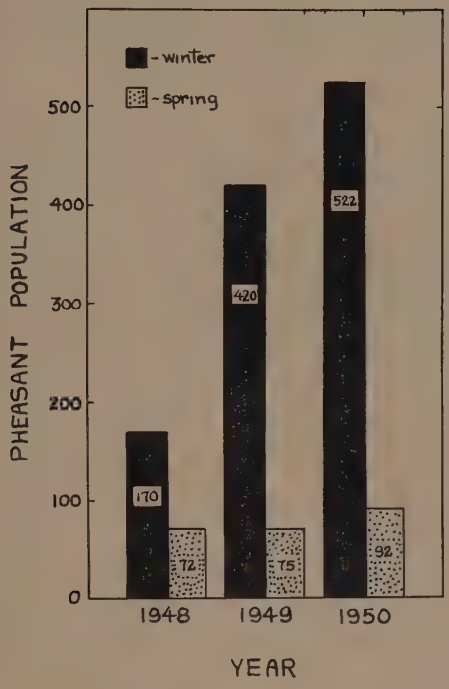

Fig. 3. Three-year, winter-spring, ring-necked pheasant populations observed at Grass Lake Game Area, Emmet County, Iowa.

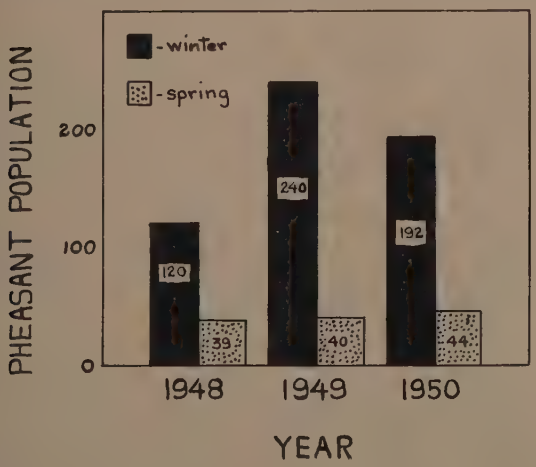

Fig. 4. Three-year, winter-spring, ring-necked pheasant populations observed at Birge Lake Game Area, Emmet County, Iowa.

An observed ratio of 37.6 males to 100 females was found on Birge Lake Area from a sample of 584 pheasants in late January and early February 1949. On Grass Lake Game Area during this same period, the observed sex ratio was 40 males to every 100 females in a sample of 694 birds.

TABLE 1

Number of Ring-necked Pheasants on the Grass Lake Game Area During Three Winters as Related to Depth of Snow on the Ground

Winter 1947 - 1948				Winter 1948 - 1949				Winter 1949 - 1950			
Date	Temp. (F.)	Snow depth (in.)	Number of birds	Date	Temp. (F.)	Snow depth (in.)	Number of birds	Date	Temp. (F.)	Snow depth (in.)	Number of birds
Feb. 21	20	Tr.	57	Jan. 8	35	2.0	142	Jan. 12	25	2.0	522
Feb. 26	35	Tr.	44	Jan. 20	3	3.0	420	Jan. 24	25	1.0	400
Mar. 1	25	2.5	99	Jan. 28	0	5.0	336	Jan. 31	-15	6.0	338
Mar. 5	22	1.0	75	Feb. 8	10	8.0	358	Feb. 8	32	Tr.	79
Mar. 8	15	8.5	170	Feb. 14	2	4.0	315	Feb. 14	15	2.0	344
Mar. 16	30	1.0	47	Feb. 19	20	Tr.	104	Feb. 26	-10	4.0	373
Mar. 26	45	0.0	34	Feb. 21	22	Tr.	262				
				Feb. 23	40	0.0	108				
				Feb. 28	10	0.0	215				
				Mar. 5	22	0.0	114				
				Mar. 10	25	0.0	110				
				Mar. 14	20	0.0	126				
				Mar. 18	25	0.0	181				
				Mar. 30	32	0.0	118				
				Mar. 31	32	1.0	215				

A ratio of 44.3 males to every 100 females was recorded at Birge on January 16, 1950 in a sample of 192 birds, while a ratio of 37.6 males to every 100 females was recorded at Grass on January 12, 1950 in a sample of 522 birds.

Each winter while driving the roads surrounding the two areas, the recorded sex ratio varied (Table 2). The presence or absence of a snow ground cover, wind, precipitation, and the time of day appeared to affect the number and sex ratio of birds observed.

Winter Cover

Cover on Birge and Grass Lakes. On both the Birge and Grass Lake Game Areas a wide variety of cover was found (Table 3). Because the two areas, with a few minor exceptions, displayed the same types of vegetative cover, the following discussion is by cover types. No significant change in the cover was noted on the two study areas during the three years of this investigation.

Uncut reed canary-grass in pure stands on both areas offered nesting cover. However, it lodged during the fall and, with the pressure of snow, became densely matted so that pheasants were seldom observed using it as winter cover.

Low vegetation, dominated by bluegrass, was present on both study areas, but it is soon matted and its value as winter cover lost.

Stubble from farm crops and cover on pasture had little value as winter cover. Pheasants frequently ranged through the stubble while feeding.

Tall, dense stands of giant ragweed provided excellent winter cover. The stalks were particularly valuable in reducing the force of the wind and snowfall. Some stalks, which were broken down by hunters during the fall or by weather during the winter, formed a protective canopy close to the ground and afforded excellent refuge and loafing cover for the birds. Where vegetation such as giant ragweed and bulrushes were bunched together, loafing and roosting cover was afforded under the resulting canopy.

Marsh vegetation such as bulrushes and cattail filled the requirements for both refuge and loafing cover but was utilized mainly and regularly by the birds for roosting as indicated by numerous forms.

TABLE 2

Observed Sex Ratio of Ring-necked Pheasants in the Estherville Area, January 9 - March 5, 1949 and 1950

Weekly periods	1949					1950			
	Males	Females	Sex unknown	Total	Sex ratio*	Males	Females	Total	Sex ratio*
Jan. 9-16	-	-	-	-	-	29	77	106	37.6
Jan. 16-22	-	-	-	-	-	81	92	173	88.9
Jan. 23-29	68	104	58	230	65.3	104	271	375	38.3
Jan. 30-Feb. 5	0	0	205	205	-	196	376	572	52.1
Feb. 6-12	191	343	191	725	55.6	228	501	729	45.5
Feb. 13-19	45	85	161	291	52.9	219	683	902	32.0
Feb. 20-26	144	196	367	707	73.4	311	742	1053	43.2
Feb. 27-Mar. 5	74	53	60	187	139.6	138	333	471	41.5
Totals	522	781	1042	2345		1306	3075	4381	

*Males per 100 females.

Corn fields were utilized mainly for feeding. In the machine-picked fields, the crushed and broken stalks offered poor cover after snow fell. Hand-picked corn provided more cover as most of the stalks remained standing all winter, but very little food was available due to the lack of waste grain in this harvesting method.

Standing, unpicked corn provided excellent winter cover, especially for feeding purposes. However, as soon as patches of bare ground began to appear through the snow cover, the pheasants ceased feeding in the standing corn almost entirely, preferring to use waste grain in the adjacent machine-picked fields.

TABLE 3

Vegetative Ground Cover on Birge Lake and Grass Lake Game Areas During the Winters of 1948, 1949 and 1950

	Acres on Grass Lake			Acres on Birge Lake		
Cover type	1947-8 winter	1948-9 winter	1949-50 winter	1947-8 winter	1948-9 winter	1949-50 winter
Canary-grass:						
stubble	-	-	-	11.5	11.5	-
uncut	8.5	8.5	8.5	16.0	12.5	12.5
Bluegrass	63.0	62.0	59.0	12.0	12.0	12.0
Giant ragweed	52.0	52.0	54.0	20.5	20.5	20.5
Mixed giant ragweed and bulrushes	-	-	-	6.5	6.5	6.5
Mixed bulrushes and cattail	28.0	28.0	28.0	-	-	-
Stubble:						
alfalfa and brome	-	-	-	18.0	18.0	18.0
brome	9.0	9.0	13.0	4.0	4.0	11.5
oats	-	4.0	-	-	-	-
barley	-	8.5	-	-	7.5	-
soybeans	8.5	-	-	19.5	5.0	10.5
Soybeans:						
unpicked	-	-	-	-	-	1.0
Field corn:						
unpicked	-	1.0	1.0	7.0	2.0	2.0
hand-picked	-	-	-	7.0	-	-
machine-picked	4.0	-	9.5	7.5	9.0	8.5
Pasture	-	-	-	7.5[a]	11.0[b]	11.0[b]
Plowed land	-	-	-	-	17.5	25.0
Totals	173.0	173.0	173.0	137.0	137.0	137.0

[a] Bluegrass

[b] Bluegrass and 3.5 acres of canary-grass

Small stands of green ash with an understory of giant ragweed were favored loafing sites during the winter. At Birge Lake Game Area, pheasants concentrated in a stand of jack pine during stormy days. Willow clumps and cottonwoods also offered protection from wind and precipitation and were utilized by the birds for loafing.

During the daylight periods when winds reached 10-12 miles per hour or snow occurred, the pheasants almost invariably were found where a mixture of six-to-eight-foot high, dense giant ragweed and eight-to-twelve foot high green ash were located. Measurements of the wind velocity in a giant ragweed stand were made on February 7, 1949, when the temperature was 20° F., and seven inches of snow covered the ground on the level.

Cover on surrounding land. Because of the intensive farming practices in Emmet County, little cover was generally available to the pheasants during the winter months. During 1948, 98 per cent of the land in Emmet County was in agricultural use.

Only a few acres were left standing by the beginning of the winter period. Fall plowing left from 30 to 45 per cent of the land bare around the two study areas during the three winters of this investigation.

The utility of many winter cover types that afforded concealment to pheasants hinged on the amount and type of snow. Dry snows reduced the concealment and protective value of cover more than wet snows. In some instances, wet snows, by accumulating on top of bulrushes and giant ragweed, furnished an effective broken canopy that at times even increased the cover value of the vegetation, but too much wet snow exerted a detrimental influence by settling the vegetation under the added weight. The efficiency of cover is best when there is no snow.

Drifting snows buried vegetative cover. Fencerows, small clumps of bushes, and ditch cover were frequently buried and thus were rendered useless as effective winter cover for pheasants. On the Birge or Grass Lake Game Area, the peripheral vegetation was buried in part by drifting snow, but the cover toward the center was protected.

While in the field during the 1948-49 winter, climatological data were collected relating to temperature, precipitation and wind velocity. The purpose was to ascertain any possible relationships between the various climatological factors and the activities of the pheasants.

Wind velocities measuring more than 10 to 12 miles per hour noticeably restricted the activity of pheasants. Green (3) came to a similar conclusion in his study of pheasants in Winnebago County, Iowa, where he found that most activity ceased when the wind reached 10 miles per hour. However, after prolonged periods of winds of 10 miles per hour or more, activities were resumed in a restricted manner. Green suggested that possibly it was not the actual velocity which influenced pheasant activity as much as it was a deviation from the normal conditions.

Temperature seemed to make little difference in the activities of the birds; although at temperatures hovering near or below zero (F.), there appeared to be some restriction.

The snow ground cover, depending on depth, appeared to limit activities to some extent. Since food was furnished on both Birge and Grass Lakes, many pheasants roosted, fed, and rested right on the areas during periods when there was more than three inches of snow cover on the ground.

When the snow cover appeared patchy, a majority of the birds still roosted on the areas but dispersed from them during the daylight hours to feed in the open fields. As the snow cover completely disappeared, many birds drifted out on surrounding land and only part of them returned to roost.

Foraging for food generally took place during the first two or three hours of the morning and the last two or three hours of the afternoon. A few individual birds were almost continually moving back and forth between the food and vegetative cover, but they were less than one per cent of the total number present.

The weather, in affecting the number of birds on the areas, also

influenced the daily movements of these same birds out from the areas. During the winter periods, pheasants ranged out from the cover on Birge and Grass Lake Game Areas to feed in the adjacent machine-picked corn fields where waste grain was to be found. Fields with recently spread manure were also frequented as were several fields containing sorghum. On days with a ten miles per hour or less wind and with a trace of or no snow ground cover, most of the pheasants ranged out to fields from one-tenth to one-half mile away.

Trapping

Trap sites. In the trapping of ring-necked pheasants on the Birge and Grass Lake Game Areas, the preliminary task was the determination of trap sites, which were based on localities most frequently used by the birds. At the Grass Lake Game Area, one trap was used in 1948, four in 1949, and five in 1950, at the Birge Lake Game Area, one in 1948, two in 1949 and 1950.

Trapping results. In 1948 pheasant traps were operated on the two study areas for 34 days between February 14 and April 4; 28 days in 1949 between January 13 and February 22 and on March 10 and April 15 to take advantage of fresh snowfalls; 57 days in 1950 between January 11 and March 15. Except for an occasional day or two, one or more traps were worked daily during these periods.

In 1948, the catch ranged from 1 to 11 in 14 of the 52 attempts. In 1949, the catch ranged from 1 to 44 birds, with a mean of 8.04 ± 8.43 in 61 of the 67 attempts. In 1950, the catch in the remaining 161 times ranged from 1 to 19 with a mean of 3.68 ± 3.49 in 161 of the 313 attempts.

A "new" bird indicates one trapped and banded for the first time. Whenever a new bird was retrapped during the same winter, it was considered a "repeat". The first time this bird was retrapped in any subsequent winter, it was a "return", while additional trappings were repeats. Any banded bird found dead in the field or shot during the hunting season was a "recovery".

TABLE 4

Results of Trapping Ring-necked Pheasants at Birge and Grass Lake Game Areas

Winter	New		Returns		Repeats		Totals		Total
	Male	Female	Male	Female	Male	Female	Male	Female	
Birge and Grass Lake Game Areas combined									
1947-48	7	37	-	-	0	6	7	43	50
1948-49	73	322	0	2	31	109	104	433	537
1949-50	87	297	3	38	28	146	118	481	599
Birge Lake Game Area									
1947-48	3	9	-	-	0	1	3	10	13
1948-49	17	68	0	1	4	10	21	79	100
1949-50	13	38	0	3	4	7	17	48	65
Grass Lake Game Area									
1947-48	4	28	-	-	0	5	4	33	37
1948-49	56	254	0	1	27	99	83	354	437
1949-50	74	259	3	35	24	139	101	433	534

During 1948, 50 pheasants were captured and released, 10 by night-shining and 40 by trapping. Forty-four of these birds were new; the remainder, repeats. In 1949, 537 pheasants were trapped: 395 were new birds; 140 were repeats; and two were returns. The 1950 trap catch totaled 599: 384 were new birds; 174 were repeats; and 41 were returns (Table 4). The trapped birds showed a lower percentage of cocks than field censuses.

Weights. Weights of 378 pheasants were accumulated between January 13 and February 19, 1949; 425, between January 11 and March 15, 1950 (Table 5). Repeats in trappings were omitted from Table 5 as these birds, with a few exceptions, were not weighed; it was possible that frequent retrapping itself reduced weight. All weights were taken while the birds were being banded and marked.

Leopold et al. (9) reported that individual pheasant weights were subject to a chance distortion up to 100 grams by reason of full or empty crops.

TABLE 5

Weights of Ring-necked Pheasants Trapped on Birge and Grass Lake Game Areas

	Jan. 13 - Feb. 19, 1949		Jan. 11 - Mar. 15, 1950	
	Male	Female	Male	Female
No. of individuals	79	299	90	335
Weight range (oz.)	34-59	20-45	34-59	28-48
Mean weight (oz.)	50.3 ± .61	36.3 ± .18	48.3 ± .41	37.1 ± .17
Standard deviation	5.5 ± .43	3.4 ± .13	3.9 ± .31	3.2 ± .12

Winter Survival

All weather factors in this study pointed toward a definite absence of emergency conditions and a corresponding relatively high survival of the pheasants. The lack of prolonged heavy snowfalls during the three winters kept the winter food supply consisting mainly of waste grains more or less readily available.

The number of pheasants that died outside of the traps on the two study areas during the 1947-48 winter period was not obtained. The remains of 26 pheasants, 14 on the Grass Lake Game Area and 12 on Birge Lake Game Area, were found during the period of January through March 5, 1949. The cause of death was determined for only one bird since an entire carcass of a bird was rarely found. In the one ascertainable case, a hen flew into a light wire and was crippled. Some undetermined predator caught her several days later. Between January 6 and March 5, 1950, the known loss of birds from undetermined causes on the two study areas totaled 12 (nine at the Grass Area and three at the Birge Area).

During the 1947-48 winter, one hen out of 50 birds entering the traps was lost. Sign at the trap indicated a farm dog. The trapping of 586

pheasants during the 1948-49 winter period resulted in injury to three birds. One female either broke or dislocated her left wing when released and was unable to fly. Two other birds, one cock and one hen, died at the time of handling. In 1949-50, seven of the 599 birds entering traps were apparently victims of farm dogs.

During early 1949, six birds seen on and near the Grass Lake Game Area were capable of sustained flight only for short distances. For instance, on January 20, a hen was seen walking very slowly with feathers fluffed out. This bird flew about 50 feet and then suddenly collapsed in mid-air and crashed to the ground. The bird was not captured.

While trapping pheasants early in 1949, 46 blood samples were taken of 11 males and 35 females and sent to the Iowa State College Veterinary Diagnostic Laboratory to be tested for the presence of Newcastle disease and pullorum. All 46 samples were negative. The catch of banded returns during the 1948-49 and 1949-50 winters indicated an average survival series of 274.3 - 20.5 - 2.0 from winter to winter for the Birge and Grass Lake Game Areas. However, these figures included only survivors returning to the traps. Additional banded survivors were known to exist in the untrapped portions of the two areas. Two hens "skipped a year" in returning to the traps.

Just how many banded birds failed to return the subsequent winter to the two areas is not known. In February and March 1950, one hen banded in 1949 was seen at a farm shelterbelt 2.0 miles from the Grass Lake Game Area.

In summary, trapped pheasants on Birge and Grass Lake Game Areas indicate a survival of banded birds represented by the series 100 - 7.4 - 0.7. The shrinkage or turnover rate of banded birds between winters on the areas was found to be from 91 to 92 per cent.

SPRING PERIOD

Spring weather. The spring of 1948 was generally moderate and was characterized, with few exceptions, by normal temperatures. The period covering late March through early June 1949, resembled the same period of 1948. During April 1950, 5 to 15°F. below normal temperatures and a 1.0 inch deficiency of precipitation occurred. This had the effect of delaying the arrival of normal spring weather from ten days to two weeks. Data on various spring phases of ring-necked pheasant behavior were gathered for the most part while driving roads surrounding Birge and Grass Lake Game Areas (Table 6).

TABLE 6

Significant Dates as Related to Ring-necked Pheasant Behavior

Activity	1948	1949	1950
Winter flock breakup	Mar. 13-19	Mar. 6-12	Mar. 6-12
Widespread crowing	Mar. 13-19	Mar. 20-26	Mar. 27-April 2
Active antagonism between cocks	Mar. 13-19	Mar. 13-19	Mar. 27-April 2
Active courtship by cocks	Mar. 13-19	Mar. 13-19	Mar. 20-26
Main spring dispersal begun	Mar. 13-19	Mar. 20-26	Mar. 20-26

Spring censuses. During each spring period of this investigation, data were gathered on the number of pheasants observed on the farmland surrounding the two areas, and in the final two spring periods, on the observed sex ratios. In addition, information was compiled on the number of birds remaining to nest on the two areas.

In arriving at the spring census totals, it was found that because of the combined factors of vegetation growth and nesting of hens the indices to the number of birds present could be best secured by counting cocks. The breeding behavior of the males inclined them to be very conspicuous in late April and early May. Knowing the number of observed cocks present and the winter observed sex ratio, it was possible to calculate an index of the total population (Table 7). An attempt was made to arrive at the spring sex ratios, but it was found to be almost impossible to calculate accurate ratios because of the great variation in the number and sex of birds recorded from week to week (Table 8).

TABLE 7

Observed Winter and Spring Ring-necked Pheasant Populations On and Off Birge and Grass Lake Game Areas

	Winter population			Spring population		
Year	On the Birge and Grass Areas	On surrounding 39 sections of land	Total	On the Birge and Grass Areas	On surrounding 39 sections of land[1]	Total
1948	290	-	-	111	483	594
1949	660	556	1216	115	877	992
1950	714	673	1387	136	881	1018

[1]1948 figure based on 24 sections.

From early april until the close of this study around June 1 of each year, the number of cocks seen per 100 hens slowly increased as more and more hens disappeared from view, presumably to nest.

In 1948, the observed nesting population on Birge Lake Game Area was 39 (15 cocks and 24 hens); at Grass Lake Game Area, 72 (22 cocks and 50 hens). By May 1, 1949, after the spring dispersal movements were completed, 40 birds (15 cocks and 25 hens) remained at Birge, and 75 (20 and 55) remained at Grass. In 1950, the observed spring population on these two areas was 44 (13 cocks and 31 hens) at Birge and 92 (27 cocks and 65 hens) at Grass. Little change was noted in the number of birds remaining to nest on the two areas from year to year even though some increase was noted from year to year in the winter populations on those areas.

The observed nesting population on sections of land surrounding Birge and Grass Lake Game Areas showed a general three-year increase in pheasants. Ingress and egress of pheasants to and from other sections caused the number to fluctuate to some extent during each spring, but the following figures were derived as an index to the population each year.

During 1948, 483 birds were observed on 24 surrounding sections of land with a mean of 20.1 birds per section. Birds varied from 4 to 26 per section. In 1949, on 39 sections, 877 birds were recorded, varying from 12 to 35 per section with a mean of 22.4 per section. In 1950, on 39 sections, 881 birds were observed with a range of 14 to 35 birds per section and a mean of 22.5.

TABLE 8

Observed Sex Ratios of Ring-necked Pheasants Off Birge and Grass Lake Game Areas During the Spring Periods of 1949 and 1950

	1949					1950			
Weekly periods	Males	Females	Sex unknown	Total observed	Sex ratio males per 100 females	Males	Females	Total observed	Sex ratio males per 100 females
Mar. 6-12	62	60	20	142	103.3	281	784	1065	35.8
Mar. 13-19	187	212	-	399	88.2	293	731	1024	40.1
Mar. 20-26	81	40	-	121	202.5	344	958	1302	35.5
Mar. 27-Apr. 2	101	145	10	256	62.6	374	748	1122	50.0
April 3-9	263	291	41	595	90.3	170	332	502	51.2
April 10-16	111	134	-	245	82.8	215	404	619	53.4
April 17-23	170	162	-	332	104.9	199	208	407	95.6
April 24-30	267	253	-	520	105.5	357	458	815	78.1
May 1-7	317	262	-	579	117.1	269	278	547	96.7
May 8-14	387	309	-	696	125.2	407	370	777	110.0
May 15-21	277	207	-	484	133.8	203	217	420	93.5
May 22-28	172	107	-	279	160.7	243	214	457	113.5
May 29-June 4	147	75	-	222	196.0	-	-	-	-

Spring mortality. The known loss of pheasants from death by various undetermined causes during the spring and early summer of 1948 included five cocks and four hens at the Grass Lake Game Area and three cocks and five hens at Birge Lake Game Area. These birds were found while systematically covering each area on foot. During the spring periods of 1949 and 1950, limited data on pheasant mortality on Birge and Grass Lakes indicated a total of three hens for each year.

MOVEMENTS

In making an investigation of the winter and spring movements of ring-necked pheasants, in 1949 and 1950, 4233 and 4870 miles, respectively, were driven over the roads surrounding the above two areas in search of marked birds. Between January 16 and June 8, 1949, 7298 pheasants were observed; between January 9 and May 28, 1950, 14,076.

In order to facilitate gathering data on movements of marked birds, the land surrounding the Birge and Grass Lake Game Areas was divided into zones circling out from the center points of those areas. Each of the first four zones extended 0.5 mile beyond the previous zone. Data were not gathered beyond 8.0 miles. This procedure implied that increased distance away from the areas was accompanied by increased areas of land to be covered. Thus the first zone, ranging from 0.0 - 0.49 miles out from the study area, included only 1.72 square miles of land while the

last zone, ranging from 7.0 - 7.99 miles out, included 49.17 square miles of land. In making observations, no single route was followed consistently, and the time spent and the mileage covered within each zone were not computed. An attempt was made to proportion the time spent in each zone according to the area of the zone.

The term cruising radius applies to the movements of the pheasants during the winter period of the study and may be regarded as synonymous with seasonal range; whereas, the term dispersal denotes the movements of pheasants without reference to direction of pheasant out from the Birge and Grass Lake Game Areas during the spring period. Since 311 of the 397 birds marked during 1949 and 371 of the 425 marked during 1950 were trapped and released on the Grass Lake Game Area, that area developed into the center of the study. Too few birds (44) were marked during the winter of 1948 to permit any extensive study of movements during that year.

No trouble was encountered in checking for banded pheasants since no banded birds had been released in north central Iowa by the Iowa State Conservation Commission for several years preceding the initiation of this study. The closest banded birds in Minnesota had been released about 30 miles north of Birge and Grass Lakes. This was considered to be too far to conflict with this investigation.

Winter Period

By March 5, 1949, 21 marked birds had been recorded off the areas. Eighteen were within 0.5 mile of Grass Lake Game Area, one within 0.5 mile of Birge Lake Game Area. On February 23, one marked cock was seen 1.0 mile east of Grass Lake. One cock was seen 0.8 mile northeast of Birge Lake Game Area on February 25. These last two were the only marked birds seen more than 0.5 mile away from the areas during the 1949 winter (Table 9).

During the winter period of January 9 to March, 1950, 141 marked pheasants, two from the Birge Lake Game Area and 139 from the Grass Lake Game Area, were recorded off the two areas. The mean distance traveled by marked birds away from Grass Lake Game Area was 0.46 mile (Table 9), and all but one were observed 1.0 mile or less from the two areas. The one exception, a cock, was observed on February 24, 2.2 miles southeast of the area. He apparently moved that distance during a period of above freezing temperatures.

In summary the January to March movements of pheasants were observed as far as 1.0 mile during 1949 and 2.2 miles in 1950, and all other observed pheasants remained within 0.8 mile of the two areas.

Spring Period

During 1949, the first sign of spring dispersal appeared during the week of March 6-12 simultaneously with the beginning of winter flock breakup. The peak of dispersal was reached during the week of April 3-9, when 48 marked birds were found from 0.5 to 3.0 miles away from the two areas. Except possibly for a few individuals the spring dispersal period was brought to a close during the week of April 24-30, at which

TABLE 9

Mean Distances Marked Ring-necked Pheasants Found Away From Birge and Grass Lake Game Areas During the Winter and Spring Periods of 1949 and 1950[1]

	Winter	Spring		
	Jan. 16 - Mar. 5	Mar. 6-31	Apr. 1-30	May 1 - June 8
Birge Area, 1949				
No. of individuals	2	14	36	12
Range in distance (mi.)	0.0-1.0	0.0-3.0	0.0-3.0	0.0-4.0
Mean distance (mi.)	-	0.57 ± .25	0.65 ± .14	2.13 ± .37
Standard deviation	-	0.95 ± .17	0.85 ± .10	1.30 ± .26
Birge Area, 1950				
No. of individuals	2	4	5	7
Range in distance (mi.)	0.0-0.4	0.0-1.3	0.0-0.7	0.0-3.7
Grass Area, 1949				
No. of individuals	19	47	97	77
Range in distance (mi.)	0.0-1.0	0.0-2.7	0.0-3.6	0.0-5.0
Mean distance (mi.)	0.52 ± .05	0.43 ± .07	1.00 ± .07	1.39 ± .11
Standard deviation	0.23 ± .03	0.49 ± .05	0.77 ± .05	1.02 ± .08
Grass Area, 1950				
No. of individuals	139	146	123	48
Range in distance (mi.)	0.0-2.1	0.0-2.2	0.0-3.2	0.0-4.3
Mean distance (mi.)	0.46 ± .02	0.55 ± .03	0.98 ± .05	1.40 ± .14
Standard deviation	0.27 ± .01	0.38 ± .02	0.65 ± .03	1.01 ± .10

[1]1950 includes period of January 9 - May 27 only.

time marked birds were found out as far as 3.7 miles. On May 10, 11 and 27, one cock and one hen were found seven miles southeast of the Grass Lake Game Area.

Spring dispersal in 1950 began during the week of March 6-12 and coincided with the breakup of winter flocks. One marked cock was found 1.8 miles east of the Grass Lake Game Area on March 7. Five to 15° F. below normal temperatures plus a nine-inch snowfall then set in, and all

movements ceased. By March 20, movements resumed, and the main spring dispersal movement developed.

The peak of the dispersal was reached in mid-April about ten days later than in 1949. By May 10, the main dispersal came to a close except for a few stray individuals.

By the end of the dispersal period, marked birds were found out as far as 4.3 miles. No birds were observed out over 4.3 miles in 1950 by the time the study was terminated on May 27.

In summarizing 1949 and 1950, a few cocks initiated spring dispersal in the week of March 6-12, although the main dispersal flow was not underway until the last ten days of March. Because of 5 to 15°F. below normal temperatures during April 1950, the peak was not reached until mid-April, a week or ten days later than the 1949 peak. Except for the movements of a few individuals, the 1950 dispersal came to a close during the first ten days of May, about ten days later than the previous year. Both years found cocks moving away first, followed by hens from ten days to two weeks later.

The greatest observed radius of spring dispersal was the same for both cocks and hens during 1949, seven miles. However, the four seven-mile records involved only one male and one female and were the exceptions, since records for 278 other marked birds were under five miles. For this reason, the seven-mile records were not included in figuring the mean distances traveled during the spring of 1949.

To facilitate easier comprehension of the annual dispersal, the means of the distances pheasants traveled away from the Birge and Grass Lake Game Areas each year were calculated in three groups (Table 9); a) records gathered between March 6 and 31, the period during which dispersal was just underway, b) data for the April 1-30 periods, gathered during the peak of the dispersal, and c) data for the remainder of the period each spring. The dispersal means for May plus part of June in 1949 and May only during 1950 were considered the most accurate as the main dispersal was concluded during this period.

Data on marked pheasants moving away from the Grass Lake Game Area were quite similar for the two years' study. In March 1949, 46 records were of birds having moved a mean distance of 0.43 miles; in March 1950, 146 records, for a mean distance of 0.55 miles. During April 1949, 96 individuals moved a mean of 1.0 miles; in April 1950, 123 birds disclosed a 0.98 mile mean. During May 1949, 80 examples were recorded with a mean of 1.39 miles; in May 1950, 47 examples, with a mean of 1.4 miles.

In Wisconsin, two reported studies of wild-reared pheasants (1 and 9) disclosed that spring dispersal movements did not extend over two miles and one and one-half miles respectively. A third study (5), made in South Dakota, reported a ten-mile extreme movement from winter to summer range. This latter movement was traced by means of crowing intensity samples and was not based on marked or banded birds.

In 1949, during May and the first week of June, following the end of dispersal in late April, 80 marked pheasants were recorded away from the Grass Lake Game Area. During May 1950, 47 were recorded off this area. Maximum distances birds had moved away from this area in different compass directions showed no relationship for 1949 and 1950.

Band Returns

Records of movements as indicated by band returns were obtained on 20 of the 675 birds banded at the Grass Lake Game Area. Late in June 1948, an incubating hen, banded the previous winter, was killed by a mower in an alfalfa field 1.5 miles northeast of that area. During the November 1948 hunting season two farmers living within 0.5 miles to the northeast of the area told of two banded males being shot on their farms.

During 1949 eight birds banded in the winter of that year were reported killed. One banded hen from the Grass Lake Game Area was killed by a mower 5.0 miles south of that area in mid-June. Seven cocks, banded on the same area, were shot during the November hunting season of 1949 and reported to the Conservation Commission. Four of these were shot on the Grass Lake Game Area, while three were killed off the area. One was shot 2.0 miles to the south, one 5.2 miles to the southeast, and one 6.2 miles to the southeast.

Following the 1950 fall hunting season, nine banded cocks were reported killed by sportsmen. All were birds banded on the Grass Lake Game Area the previous winter. Five were killed on the area where they were banded, and one was shot about six miles south near Gruver, Iowa. The three other banded returns contained no data on the location where the birds were killed. Of the 167 male birds that were banded during the three-year study, 16 (9.0 per cent) bands were returned by sportsmen.

SUMMARY

The purpose of this investigation was to contribute information on the winter and spring movements of wild-reared ring-necked pheasants. Data were gathered in the Estherville Area, including parts of both Emmet County, Iowa, and adjacent Martin County, Minnesota. Observations were centered around two sites known for winter concentrations of pheasants, Birge and Grass Lake Game Areas.

Trapping was undertaken in order to mark pheasants for subsequent study of their movements. Pheasants were marked for identifying purposes with two leg bands and by painting tail feathers.

The maximum winter populations observed on the Grass Lake Game Area were 170 in 1948; 420 in 1949; and 522 in 1950. A count of 120 on the Birge Lake Game Area in 1948 increased to 240 in 1949, but dropped to 192 in 1950. The observed winter sex ratio in the field during these three years ranged from 37.6 to 50.0 males per 100 females.

Correlations were found between the number of birds recorded on the Grass Lake Game Area and the depth of snow on the ground. Pheasant activity during the winter months was restricted during periods when winds measured more than 10 to 12 miles per hour.

The two game areas with their variety of vegetative cover helped fill the need for winter pheasant cover in the Estherville Area. The most heavily utilized types of cover present were giant ragweed, river bulrush, green ash, white and peachleaf willows and cottonwoods. Farm shelterbelts were most frequently relied on by the pheasants for winter cover off the areas.

Trapping was conducted on the Birge and Grass Lake Game Areas during each of the three winter periods covered in this investigation. The most successful trap sites were located in stands of mixed giant ragweed and green ash and in a small stand of young white willows.

During 1948, 50 pheasants were captured, including 44 new birds and six repeats. In 1949, 537 were trapped, including 395 new birds, 140 repeats and two returns from 1948. In 1950, 599 birds were trapped, of which 384 were new birds, 174 were repeats, and 41 were returns, 39 from 1949 and two from 1948.

Weights of 378 pheasants (79 cocks and 299 hens) were recorded during the 1949 trapping season and 425 (90 cocks and 335 hens) during 1950. Mean weights for cocks were 50.3 ounces in 1949 and 48.3 ounces in 1950. Mean weights for hens were 36.3 ounces in 1949 and 37.1 ounces in 1950.

Three consecutive trappings of pheasant populations wintering on Birge and Grass Lake Game Areas indicated a survival of banded birds represented by the series of 100 - 7.4 - 0.7. The shrinkage or turnover rate of banded birds on the areas between winters was found to be from 91 to 92 per cent.

The observed number of pheasants that remained to nest on the two areas each year showed little change even though observed maximum winter populations showed rather pronounced increases. Annual nesting populations on the Birge Lake Game Area varied between 39 in 1948 and 44 in 1950. The nesting population on the Grass Lake Game Area varied from 72 in 1948 to 92 in 1950. Observed nesting populations on 39 sections of farm land surrounding the two areas varied from 12 to 35 birds per section, with means of 20.1 per section in 1948, 22.4 in 1949 and 22.5 in 1950.

During the 1949 winter period, all but one of 21 marked birds observed off Birge and Grass Lake Game Areas were within a radius of 1.0 mile. The mean distance traveled by 19 pheasants out from the Grass Lake Game Area was 0.52 mile. One hundred and forty-six marked birds observed off the two areas during the winter of 1950 were all within 1.0 mile of the areas, except for one cock seen 2.16 miles from the Grass Lake Game Area. Mean distance traveled by 139 birds from the Grass Lake Game Area was 0.46.

Each year during the spring period, from 70 to 83 per cent of the birds wintering on the two areas dispersed onto the surrounding farm land.

The first dispersal movements were recorded early in March of each year of this investigation. Main dispersal was underway between March 13-19 in 1948 and between March 20-26 in 1949 and 1950. The peak of dispersal movements was reached during the week of April 3-9 in 1949 but in mid-April in 1950. The last week in April brought main dispersal movements to a conclusion in 1949, while May 10 brought a general termination to the 1950 movements.

The greatest observed radius of spring dispersal of marked pheasants was the same for both sexes during 1949, 7.0 miles. During 1950, no movement was observed over 4.3 miles.

Forty-six marked birds moved a mean distance of 0.43 mile from the Grass Lake Game Area in March 1949. The mean for 146 observations in March 1950 was 0.55 mile. Means for 95 records and 123 records

gathered during the peak of dispersal in April of each year were 1.00 mile in 1949 and 0.98 in 1950.

The means for data gathered in May of each year at the conclusion of spring movements out from Grass Lake found each year's dispersal comparable. During 1949, 80 examples had a mean of 1.39 miles; during 1950, 47 had a mean of 1.40 miles.

Each spring, aside from a few exceptions, observed marked pheasants dispersed no more than 4.5 to 5.0 miles out on the farm land surrounding the two areas.

Records of movements, as indicated by band returns, were obtained on 20 of the 675 birds banded at the Grass Lake Game Area. In 1948 the records included one hen (1.5 miles) and two cocks (0.5 mile each). The 1949 reports included one hen (5.0 miles) and seven cocks (four, no movement and three, 2.0, 5.2, and 6.2 miles respectively). Nine banded cocks were reported killed in 1950: five on the area, one 6.0 miles away, the other four had no data of location of kill. Of the 167 male birds banded during the three-year study, 16 (9.0 per cent) bands were returned by sportsmen.

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THE FALL POPULATION EUROPEAN CORN BORER SURVEY[1]

T.A. Bancroft, T.A. Brindley, Robert Bowles, and P.C. Tang[2]

Iowa State College, Ames, Iowa

I. INTRODUCTION

The objects of this paper are: (i) to discuss two estimation methods used in the Fall Population European Corn Borer Survey as to possible biases and to present a formula for the estimated variance or estimated mean square error, as the case may be, for each; (ii) using the formulas from (i) to present some numerical results based on data from 12 districts covering the state of Iowa for the years 1949, 1950, and 1951.

The Survey is begun after the first killing frost so that all growth of the corn plants is stopped. The state is divided into 12 districts, numbered I - XII on maps provided for the enumerators. Fifty stops are selected at random in each district and marked 1 - 50 on the maps. The enumerator is instructed to locate a stop by prejudging the distance of a stop number on the map from the nearest town, highway junction, etc. He is directed to drive to the stop as identified by the speedometer reading from the end of the "speed zone" of the located nearest town or from the located highway junction, etc. He is asked to take the field nearest to such a stop, using the one on the right side of the road in case there are fields on both sides. He is instructed to walk 25 paces into the field from near the middle of the most accessible edge. He is to begin with the first plant on his right, make a mark of identification and count 25 consecutive plants. The 25 plants are to be examined as he returns to his mark and certain data recorded which include the number of "infested" plants; for the i^{th} selected sampling unit, m_i; out of the 25. He is instructed to dissect the last two "infested" plants in the 25 examined, recording the number of borers for each of the two plants, and the mean number of borers per "infested" plant, $\bar{y}_i$, for the i^{th} selected sampling unit.

An "infested" plant is defined as a plant with entrance holes or some other definite evidence of the presence of the borer on the plants at time of inspection or in the past. Since one borer may make several entrance holes, there is not necessarily a different borer for each entrance hole. Also, since all borers in the plant may have been destroyed by parasitic or predatory insects or in other ways, an "infested" plant upon dissection

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[2] Director of the Statistical Laboratory, Entomologist, Iowa Agricultural Experiment Station, Research Associate, and Associate Professor of Statistics, Iowa State College, respectively. T. A. Brindley is also connected with the Bureau of Entomology and Plant Quarantine, United States Department of Agriculture.

may contain no borers. Furthermore, at the time of dissection the borers may have completed their life cycle and emerged as moths. For a plant not "infested" it is assumed certain that there are no borers.

The enumerator records the proportion of infested plants, $w_i = \frac{m_i}{25}$ on the i^{th} selected sampling unit.

The above sampling procedure[1] was used by entomologists of the Iowa Department of Agriculture, Experiment Station and Extension Service under the direction of Dr. H. M. Harris, Iowa State Entomologist and Head of the Department of Zoology and Entomology at Iowa State College. It was based on techniques used by the Bureau of Entomology and Plant Quarantine, United States Department of Agriculture, and cooperating States since 1939 in determining fall populations of the European corn borer.

The method described above of selecting the two "infested" plants to be dissected is simple and can be quickly performed; however, since it may introduce a bias, it is recommended that the two "infested" plants be selected at random from the m_i "infested" plants in the i^{th} selected sampling unit. This may be done fairly easily by providing the enumerator in advance with a sheet of random selections of 2 out of numbers from 3 to 25. (If there are none "infested" in the 25 examined, no dissection is made; if only one is infested, then that one only is dissected. If 2 are "infested", then both plants are dissected.) It is true of course that to obtain random selections it will be necessary for the enumerator to identify and number the "infested" plants in each sampling unit before locating the 2 selected at random by use of the sheet of random selections. All analyses in this investigation will assume a random selection of the 2 plants.

II. ESTIMATION PROCEDURES

Two methods have been proposed for estimating the mean number of borers per plant for each district. The first method is

$$(A) \qquad \bar{y} = \frac{1}{n} \sum_{i=1}^{n} w_i \bar{y}_i$$

where

n = number of sampling units,

w_i = the proportion of infested plants on the i^{th} selected sampling unit,

$\bar{y}_i$ = the mean number of borers per infested plant for the i^{th} selected sampling unit obtained by dissecting the 1 or 2 infested plants.

[1] Some uses of modern survey sampling techniques involving the use of the analysis of variance in estimating the fall population of the European corn borer were presented by Meyers and Patch (2) as early as 1937.

It is shown in a technical appendix that this estimator is unbiased and the estimated variance is given by

$$\hat{V}_{\bar{y}} = \frac{1}{n(n-1)} \left\{ \sum_{i=1}^{n} (w_i \bar{y}_i)^2 - \frac{\left(\sum_{i=1}^{n} w_i \bar{y}_i \right)^2}{n} \right\}$$

$$= \frac{1}{(25)^2 n(n-1)} \left[\sum_{i=1}^{n} (m_i \bar{y}_i)^2 - \frac{\left(\sum_{i=1}^{n} m_i \bar{y}_i \right)^2}{n} \right]$$

where we have assumed the finite population of sampling units so large that it may be considered infinite.

As mentioned above this estimator is unbiased, also the estimated variance is simple to calculate.

Since it is possible for an "infested" plant to yield zero borers upon dissection, it would be possible for a sampling unit to have up to 25 "infested" plants, i.e. $w_i = 1$, and yet the two plants selected for dissection might give $\bar{y}_i = 0$. In such a case, the information on the weight, w_i, would be lost in using the estimator (A). For this reason a second estimator has been proposed, namely,

$$(B) \qquad \bar{y}' = \left(\frac{1}{n} \sum_{i=1}^{n} w_i \right) \left(\frac{1}{n} \sum_{i=1}^{n} \bar{y}_i \right)$$

for which no information is lost on the w_i's except in the most unusual case where the second factor is zero.

It is shown in the technical appendix that estimator (B) is biased however, the population bias being given by

$$\beta = -\frac{n-1}{n} \rho_{w_i \bar{Y}_i} \sigma_{w_i} \sigma_{\bar{Y}_i}$$

where $\bar{Y}_i$ is the actual mean number of borers on the i^{th} selected sampling unit, and again we have assumed that the finite population of sampling units is so large that it may be considered infinite. In case w_i and $\bar{Y}_i$ are positively correlated, the bias will be downwards and $\bar{y}'$ will tend to underestimate the population mean number of borers per plant. This bias does not approach zero as the size of the sample is increased, but will be zero only if some one or all of its factors is or are zero. An estimate of the bias can be obtained from

$$\hat{\beta} = \bar{y}' - \bar{y} .$$

Now, the mean square error for estimator (B) is

$$(MSE)_{\bar{y}'} = V_{\bar{y}'} + \beta^2,$$

which is estimated by

$$(\widehat{MSE})_{\bar{y}'} = \hat{V}_{\bar{y}'} + \hat{\beta}^2.$$

It is shown in the technical appendix that

$$(\widehat{MSE})\ \bar{\bar{y}}' = \hat{V}_{\bar{\bar{y}}} + \hat{\Delta}.$$

where

$$\hat{\Delta} = \left(1 + \frac{6}{n^2}\right) m^2_{11(w_i\bar{y}_i)} - \frac{2}{n-2}\left(\bar{\bar{w}}\, m_{12(w_i\bar{y}_i)} + \bar{\bar{y}}\, m_{21(w_i\bar{y}_i)}\right)$$

$$+ \frac{3}{n^2}\, m_{20(w_i\bar{y}_i)}\ m_{02(w_i\bar{y}_i)} - \frac{n+4}{n^2}\, m_{22(w_i\bar{y}_i)},$$

neglecting terms involving $\frac{1}{n^3}$ and disregarding covariances between w_i and $m_{12}(w_i\bar{y}_i)$, etc. We define $\bar{\bar{y}}$ to be the mean of the $\bar{y}_i$'s.

III. EMPIRICAL RESULTS

Tables I, II, and III present summarizing results using formulas from section II for the 12 districts for Iowa for each of the years 1949, 1950, and 1951.

TABLE I (n = 50)

1949 Fall Survey Data (Heavy Infestation)

	$\bar{\bar{y}}$	$\bar{\bar{y}}'$	$\hat{\beta}$	$\hat{V}_{\bar{\bar{y}}}$	$(\widehat{MSE})\ \bar{\bar{y}}'$	$\widehat{R.E.}(\bar{\bar{y}}$ to $\bar{\bar{y}}')$
I	15.4128	15.5598	0.1470	1.8276	1.8379	1.0056
II	11.8300	11.8300	0.0000	0.6993	0.6993	1.0000
III	7.5660	7.4784	-0.0876	1.0760	1.0729	0.9971
IV	9.7408	9.7387	-0.0021	0.8884	0.8857	0.9970
V	11.2256	11.1257	-0.0999	1.2796	1.2933	1.0107
VI	6.8456	6.7234	-0.1222	0.7697	0.7793	1.0124
VII	9.8744	9.7950	-0.0794	1.0628	1.0664	1.0033
VIII	7.4408	7.4142	-0.0266	0.4891	0.4890	0.9998
IX	3.0100	2.9417	-0.0683	0.0962	0.0935	0.9720
X	4.8564	4.4521	-0.4043	0.5624	0.6670	1.1861
XI	4.4496	4.2791	-0.1705	0.4275	0.4433	1.0372
XII	2.5256	2.3218	-0.2038	0.1612	0.1919	1.1902
Overall Mean	7.8981	7.8050	-0.0931	0.7783	0.7933	1.0343

Table II (n = 50)

1950 Fall Survey Data (Light Infestation)

	$\bar{y}$	$\bar{y}'$	$\hat{\beta}$	$\hat{V}\bar{y}$	$(\widehat{MSE})\ \bar{y}'$	$\widehat{R.E.}(\bar{y}$ to $\bar{y}')$
I	3.8308	3.5572	-0.2736	0.1966	0.2922	1.4869
II	1.1372	1.0491	-0.0881	0.0276	0.0256	0.9270
III	1.2408	1.1270	-0.1138	0.0366	0.0380	1.0374
IV	3.1736	3.0717	-0.1019	0.1282	0.1380	1.0765
V	1.4248	1.3276	-0.0972	0.0399	0.0480	1.2038
VI	1.0068	0.9218	-0.0850	0.0219	0.0224	1.0208
VII	2.0652	2.0448	-0.0204	0.0997	0.1026	1.0295
VIII	0.6060	0.5748	-0.0312	0.0095	0.0073	0.7721
IX	0.2364	0.1935	-0.0429	0.0017	0.0031	1.8224
X	0.7912	0.6998	-0.0914	0.0113	0.0175	1.5562
XI	0.5988	0.5076	-0.0912	0.0121	0.0161	1.3261
XII	0.2780	0.2599	-0.0181	0.0017	0.0019	1.1466
Overall Mean	1.3658	1.2779	-0.0879	0.0489	0.0594	1.2004

The year 1949 was considered a heavy infestation year, for the three year's of data, by the entomologists. The overall mean number of borers per plant being estimated as 7.9 or 7.8. Now, if the infestation is consistently heavy, then it is evident from the formulas that $w_i \longrightarrow 1$ and $\bar{y}' \longrightarrow \bar{y}$. This is borne out by the empirical results of Table I, also the overall estimated relative efficiency of the estimator A to the estimator B, of $\widehat{R.E.}(\bar{y}$ to $\bar{y}') = 1$. In Table I all districts except I and II have negative estimated biases for $\bar{y}'$ as was to be expected when the correlation between w_i and $\bar{y}_i$ is positive. In district II all plants in each sampling unit of 25 were infested, hence $\bar{y} = \bar{y}' = 11.8300$, and $\widehat{R.E.}(\bar{y}$ to $\bar{y}') = 1$. In district I the estimated bias is positive. For district I the values of w_i were almost all 1, and the estimated correlation of w_i and $\bar{y}_i$ is probably not very reliable.

The year 1950, results summarized in Table II, was considered a light infestation year, for the 3 consecutive years, by the entomologists. The overall mean number of borers per plant being estimated as 1.4 or 1.3. For this year, the overall mean of $\widehat{R.E.}(\bar{y}$ to $\bar{y}')$ is 1.2. In Table II all districts have negative estimated biases for $\bar{y}'$ as was to be expected.

TABLE III (n = 50)

1951 Fall Survey Data (Very Light Infestation)

	$\bar{y}$	$\bar{y}'$	$\hat{\beta}$	$\hat{V}\bar{y}$	$(\widehat{MSE})\ \bar{y}'$	$\widehat{R.E.}(\bar{y}$ to $\bar{y}')$
I	0.4980	0.4269	-0.0711	0.0076	0.0078	1.0177
II	1.5596	1.4616	-0.0980	0.0667	0.0708	1.0617
III	0.3156	0.2930	-0.0226	0.0042	0.0034	0.8172
IV	0.6412	0.5457	-0.0955	0.0093	0.0160	1.7307
V	0.2508	0.2232	-0.0276	0.0033	0.0030	0.8985
VI	0.2736	0.2565	-0.0171	0.0019	0.0025	1.3008
VII	1.7180	1.5682	-0.1498	0.0779	0.0867	1.1125
VIII	0.9232	0.7169	-0.2063	0.0280	0.0600	2.1428
IX	0.1980	0.1329	-0.0651	0.0026	0.0053	2.0480
X	1.3164	1.1579	-0.1585	0.0409	0.0553	1.3528
XI	0.8684	0.6572	-0.2112	0.0356	0.0613	1.7208
XII	0.3472	0.2312	-0.1160	0.0064	0.0134	2.1048
Overall Mean	0.7425	0.6393	-0.1032	0.0237	0.0321	1.4424

The year 1951, results, summarized in Table III, was considered a very light infestation year for the 3 consecutive years, by the entomologists, the overall mean number of borers per plant being estimated as 0.7 or 0.6. The overall mean of $\widehat{R.E.}(\bar{y}$ to $\bar{y}')$ is 1.4.

IV. DISCUSSION

For the 1949 data (Heavy Infestation) not only was the overall mean of the $\widehat{R.E.}(\bar{y}$ to $\bar{y}')$ approximately equal to 1, but this was also true for each individual district. Hence, for this year, it matters little whether we use $\bar{y}$ or $\bar{y}'$.

For the 1950 data (Light Infestation), except for districts II and VIII, $\bar{y}$ has the smaller estimated variation and on the basis of this evidence would be preferred.

A statement similar to that made for the 1950 data may be made for the 1951 data. (Very Light Infestation)

If one is permitted to speak of a trend for only 3 years of data, it would appear that these empirical results bear out what one might expect,

i.e. that as the infestation becomes lighter, $\bar{y}$ tends to become a more exact estimator than $\bar{y}'$, although there are a few exceptions.

On the bases of these empirical studies only one might make the following recommendations; (i) for a Heavy Infestation year either $\bar{y}$ or $\bar{y}'$ will work equally well. (ii) for a Light or Very Light Infestation year, $\bar{y}$ would be preferred. However, since the variance of $\bar{y}$ is an exact statistical expression and easy to calculate, and our empirical study so far shows no real consistent reason for ever preferring $\bar{y}'$, the present recommendation would seem to be to use $\bar{y}$. Of course, the curious statistician or biometrician would probably try both.

V. FURTHER CONSIDERATIONS

Some thought has been given to the problem of extending the use of the double sampling method employed. Instead of assigning 0 or 1 to a plant according as to whether it is not "infested" or is "infested", it is proposed to obtain more refined measures of this second variate, on which observations are cheap to obtain. That is, it is proposed that a study be made of the ability of an entomologist to give eye estimates of the degree of infestation, say, 0, 1, 2, 3. Should these eye estimates prove satisfactory and a fairly high correlation be found to exist between this variate and the mean number of corn borers per infested plant, it should be possible to use the first variate in the usual double sampling scheme to improve the estimate desired. See Cochran (3).

VI. TECHNICAL APPENDIX

Let* M_i and $W_i = \frac{M_i}{25}$, $(i = 1,2, \ldots, N)$, be the number and proportion of infested plants in the i^{th} sampling unit from the district; M_i', W_i', $(i = 1,2, \ldots, n)$, be those for the i^{th} selected unit. Y_{ij}, $(j = 1,2, \ldots, M_i$; $i = 1,2, \ldots, N)$, be the number of corn borers in the j^{th} infested plant from the i^{th} unit in the same district; y_{ij}, $(j = 1, 2$ if $M_i' \geq 2$; $j = 0$ or 1 if $M_i' = 0$ or 1 respectively; $i = 1,2, \ldots, n)$ be that in the j^{th} selected infested plant from the i^{th} selected unit.

For the population considered we have

$$\bar{Y}_{i.} = \frac{1}{M_i} \sum_{j}^{M_i} Y_{ij}$$

the average number of corn borers per infested plant in the i^{th} unit;

$$\bar{\bar{Y}} = \frac{1}{N} \sum_{i}^{N} \bar{Y}_{i.}$$

* Some of the symbols used in this section are slightly different from those used in the main thesis. These are necessary to insure the exactness during the course of derivation of the formulas.

the mean of the average number of corn borers per infested plant per unit;

$$\overline{W} = \frac{1}{N} \sum_{1}^{N} W_i ,$$

the average proportion of infested plants per unit.

$$\overline{Y} = \frac{1}{25N} \sum_{1}^{N} M_i \overline{Y}_{i\cdot} = \frac{1}{N} \sum_{1}^{N} W_i \overline{Y}_{i\cdot} = \frac{1}{25N} \sum_{1}^{N} \sum_{j}^{M_i} Y_{ij} ,$$

the average number of corn borers per plant, the quantity to be estimated.

From a sample obtained we can calculate

$$\overline{y}_i = \frac{1}{m_i} \sum_{j}^{m_i} y_{ij} \; ; \quad \overline{y}_i = 0, \text{ if } m_i = M_i = 0.$$

$$\overline{\overline{y}} = \frac{1}{n} \sum_{1}^{n} \overline{y}_i .$$

$$\overline{w} = \frac{1}{n} \sum_{1}^{n} W_i' .$$

The proof of the results required will be simplified when the expectation of covariance of different orders for sample means of two characteristics is worked out first. We give the following results without proof:

If

$$E(u) = E(v) = 0, \quad \operatorname{cov}(u^r v^s) = E(u^r v^s) = \mu_{rs} \; ;$$

$\overline{u}$ and $\overline{v}$ be the means of the characteristics u and v from a random sample of n selected from a population of N, then

(1) $$E(\overline{u}\,\overline{v}) = (e_1 - e_2) \frac{N\mu_{11}}{n^2} = \frac{N-n}{N-1} \frac{\mu_{11}}{n} \longrightarrow \frac{\mu_{11}}{n} , \text{ when N is large;}$$

(2) $$E(\overline{u}^2) = (e_1 - e_2) \frac{N\mu_{20}}{n^2} = \frac{N-n}{N-1} \frac{\mu_{02}}{n} \longrightarrow \frac{\mu_{20}}{n} \; ;$$

(3) $$E(\overline{u}\,\overline{v}^2) = (e_1 - 3e_2 + 2e_3) \frac{N\mu_{12}}{n^3} \longrightarrow \frac{\mu_{12}}{n^2}$$

(4) $$E(\overline{u}\,\overline{v}^3) = (e_1 - 7e_2 + 12e_3 - 6e_4) \frac{N\mu_{13}}{n^4} + 3 (e_2 - 2e_3 + e_4) \frac{N^2\mu_{11}\mu_{02}}{n^4}$$

$$\longrightarrow \frac{\mu_{13}}{n^3} + \frac{3(n-1)}{n^3} \mu_{11}\mu_{02} \; ;$$

$$(5)\quad E(\bar{u}^2\bar{v}^2) = (e_1 - 7e_2 + 12e_3 - 6e_4)\frac{M\mu_{22}}{n^4} + (e_2 - 2e_3 + e_4)$$

$$(2\mu_{11}^2 + \mu_{20}\mu_{02})\frac{M^2}{n^4} \longrightarrow \frac{\mu_{22}}{n^3} + \frac{n-1}{n^3}(2\mu_{11}^2 + \mu_{20}\mu_{02}) \quad ;$$

where $e_i = \dfrac{n(n-1)\ \ldots\ (n-i+1)}{N(N-1)\ \ldots\ (N-i+1)}$.

Consider the estimator A: $\bar{\bar{y}} = \frac{1}{n}\sum_i^n W_i'\ \bar{y}_i$.

Let E_{1i} be the operation for the expectation for fixed i,

$$\bar{y}_{i\cdot} = \frac{1}{M_i'}\sum_j^{M_i'} y_{ij} \quad ; \quad \bar{y}_{i\cdot} = 0, \text{ if } M_i' = 0.$$

$$\bar{\bar{y}} = \frac{1}{n}\sum_i^n \bar{y}_{i\cdot}$$

Then

$$E_{1i}(\bar{y}_{i\cdot}) = \bar{y}_{i\cdot} \quad , \quad E(\bar{\bar{y}}) = \frac{1}{n}\sum_i^n E_{1i}(\bar{y}_{i\cdot}) = \frac{1}{n}\sum_i^n \bar{y}_{i\cdot} = \bar{\bar{y}}.$$

The expectation of $\bar{\bar{y}}$ is

$$(6)\quad E(\bar{\bar{y}}) = E\left[\frac{1}{n}\sum_i^n W_i' E_{1i}(\bar{y}_{i\cdot})\right] = E\left(\frac{1}{n}\sum_i^n W_i'\bar{y}_{i\cdot}\right) = \frac{1}{N}\sum_i^N W_i \bar{Y}_{i\cdot} = \bar{Y} \quad .$$

$\bar{\bar{y}}$ is an unbiased estimate of $\bar{Y}$.

The variance of $\bar{\bar{y}}$ is

$$(7)\quad V(\bar{\bar{y}}) = E(\bar{\bar{y}} - \bar{Y})^2 = \frac{1}{n^2}\left\{ E\left[\sum_i^n W_i'(\bar{y}_{i\cdot} - \bar{y}_{i\cdot})\right]^2 + E\left[\sum_i^n (W_i'\bar{y}_{i\cdot} - \bar{Y})\right]^2 \right\}$$

$$= \left(\frac{1}{n} - \frac{1}{N}\right)\sigma_b^2 + \frac{1}{n^2}\ E\left[\sum_i^n W_i'^2 E_{1i}(\bar{y}_{i\cdot} - \bar{y}_{i\cdot})^2\right]$$

$$= \left(\frac{1}{n} - \frac{1}{N}\right)\sigma_b^2 + \frac{1}{nN}\sum_i^N W_i^2\left(\frac{1}{m_i} - \frac{1}{M_i}\right)\sigma_i^2 \quad ,$$

where

$$\sigma_b^2 = \frac{1}{N-1}\sum_i^N (W_i\bar{Y}_{i\cdot} - \bar{Y})^2 \quad , \quad \sigma_i^2 = \frac{1}{M_i - 1}\sum_j^{M_i}(Y_{ij} - \bar{Y}_{i\cdot})^2 \quad .$$

To get the estimate of $V(\bar{\bar{y}})$, denote

$$s_i^2 = \frac{1}{m_i - 1} \sum_j^{m_i} (y_{ij} - \bar{y}_i)^2 , \quad s_i^2 = 0 \text{ if } m_i = M_i = 0 \text{ or } 1 ;$$

$$s_b^2 = \frac{1}{n-1} \sum_i^n (W_i' \bar{y}_i - \bar{\bar{y}})^2 .$$

Evidently

$$E(s_i^2) = \sigma_i^2 ,$$

$$E(s_b^2) = \frac{1}{n-1} E\left\{ \sum \left[W_i' (\bar{y}_i - \bar{y}_{i.}) + (W_i' \bar{y}_{i.} - \bar{\bar{Y}}) - (\bar{\bar{y}} - \bar{\bar{Y}}) \right]^2 \right\}$$

$$= \frac{1}{n-1} \left\{ \frac{n}{N} \sum_i^N W_i^2 \left(\frac{1}{m_i} - \frac{1}{M_i}\right) \sigma_i^2 + \frac{n}{N} \sum_i^N (W_i \bar{Y}_i - \bar{\bar{Y}})^2 - V(\bar{\bar{y}}) \right\} ,$$

or

$$(8) \quad (n-1)\, s_b^2 = \frac{n}{N} \sum_i^N W_i^2 \left(\frac{1}{m_i} - \frac{1}{M_i}\right) \sigma_i^2 + \frac{n(N-1)}{N} \sigma_b^2 - V(\bar{\bar{y}}) .$$

Eliminating σ_b^2 between (7) and (8), we obtain the estimate of $V(\bar{\bar{y}})$

$$(9) \quad \hat{V}(\bar{\bar{y}}) = \left(\frac{1}{n} - \frac{1}{N}\right) s_b^2 + \frac{1}{nN} \sum_i^n W_i'^2 \left(\frac{1}{m_i} - \frac{1}{M_i'}\right) s_i^2$$

$$\longrightarrow \frac{1}{n} s_b^2 = \frac{1}{n(n-1)} \left\{ \sum_i^n (W_i' \bar{y}_i)^2 - \frac{\left(\sum_i^n W_i' \bar{y}_i\right)^2}{n} \right\} ,$$

when N is large. The last expression is useful for computation.

Turn, now, to the estimator B:

$$\bar{\bar{y}}' = \left(\frac{1}{n} \sum_i^n W_i'\right) \left(\frac{1}{n} \sum_i^n \bar{y}_i\right) = \bar{w}\, \bar{\bar{y}} .$$

Let $\delta\bar{w}$, $\delta\bar{\bar{y}}$ be the deviations of $\bar{w}$, $\bar{\bar{y}}$ from their expectations $\bar{W}$, $\bar{\bar{Y}}$ respectively, and denote

$$\mu_{rs}(W_i \bar{Y}_{i.}) = \frac{1}{N} \sum_i^N (W_i' - \bar{W})^r (\bar{Y}_{i.} - \bar{\bar{Y}})^s ,$$

$$\mu_{rs}(W_i V_i) = \frac{1}{N} \sum_i^N (W_i - \bar{W})^r (V_i - \bar{V})^s ,$$

where

$$V_i = \left(\frac{1}{m_i} - \frac{1}{M_i}\right)\sigma_i^2 \quad , \quad \bar{V} = \frac{1}{N}\sum_i^N \left(\frac{1}{m_i} - \frac{1}{M_i}\right)\sigma_i^2 \quad .$$

The expectation of $\bar{y}'$ is

$$E(\bar{y}') = E(\bar{w}\,\bar{\bar{y}}) = E(\delta\bar{w} + \bar{W})\,(\delta\bar{\bar{y}} + \bar{\bar{Y}}) = E(\delta\bar{w}\,\delta\bar{\bar{y}}) + \bar{W}\,\bar{\bar{Y}} \quad .$$

Apply (1) and note

$$\bar{W}\,\bar{\bar{Y}} = \frac{1}{N}\left[\sum_i^N W_i\,\bar{Y}_i - \sum_i^N (W_i - \bar{W})\,(\bar{Y}_i - \bar{\bar{Y}})\right] = \bar{\bar{Y}} - \mu_{11(\bar{Y}_{i\cdot})} \quad ,$$

we get

$$(10)\quad E(\bar{y}') = \bar{\bar{Y}} - \mu_{11(W_i\bar{Y}_{i\cdot})} + \frac{N-n}{N-1}\,\frac{\mu_{11(\bar{Y}_{i\cdot})}}{n} = \bar{\bar{Y}} - \frac{n-1}{n}\,\frac{N}{N-1}\,\mu_{11(W_i\bar{Y}_{i\cdot})}$$

The estimator is biased with the bias

$$(11)\quad \beta = -\frac{n-1}{n}\,\frac{N}{N-1}\,\mu_{11(W_i\bar{Y}_{i\cdot})} \longrightarrow -\frac{n-1}{n}\,\rho_{W_i\bar{Y}_{i\cdot}}\,\sigma_{W_i}\,\sigma_{\bar{Y}_{i\cdot}} \quad ,$$

when N is large.

The mean square error of $\bar{y}'$ is

$$(12)\quad MSE(\bar{y}') = E(\bar{y}' - \bar{\bar{Y}})^2 = V(\bar{y}') + \beta^2 \quad ,$$

$$= E\left[\bar{w}^2\,(\bar{\bar{y}} - \bar{\bar{y}}_{\cdot})^2\right] + E(\bar{w}^2\,\bar{\bar{y}}_{\cdot}^2) - \left[E(\bar{w}\,\bar{\bar{y}}_{\cdot})\right]^2 + \beta^2$$

As

$$E_{1i}\,(\bar{\bar{y}} - \bar{\bar{y}}_{\cdot})^2 = \frac{1}{n^2}\sum_i^n \left(\frac{1}{m_i} - \frac{1}{M_i'}\right)\sigma_i^2 = \frac{1}{n}\,\bar{v}, \quad \text{where}$$

$$\bar{v} = \frac{1}{n}\sum_i^n \left(\frac{1}{m_i} - \frac{1}{M_i'}\right)\sigma_i^2 \quad .$$

$$(13)\quad E\left[\bar{w}^2\,(\bar{\bar{y}} - \bar{\bar{y}}_{\cdot})^2\right] = \frac{1}{n}\,E(\bar{w}^2\,\bar{v}) = \frac{1}{n}\,E\left[(\delta\bar{w} + \bar{w})^2\,(\delta\bar{v} + \bar{v})\right]$$

$$\longrightarrow \frac{1}{n}\left\{\bar{W}^2\,\bar{V} + \frac{\bar{V}}{n}\,\mu_{20(W_i\bar{Y}_{i\cdot})} + \frac{\bar{W}}{n}\,\mu_{11(W_i\bar{V}_{i\cdot})} + \frac{1}{n^2}\,\mu_{21(W_i\bar{V}_i)}\right\} \quad .$$

after expanding, applying (1), (2), (3), and assuming N large.

Similarly,

$$(14)\quad E(\bar{w}^2\,\bar{\bar{y}}_{\cdot}^2) - \left[E(\bar{w}\,\bar{\bar{y}}_{\cdot})\right]^2 \longrightarrow \frac{1}{n}\left[\bar{W}^2\,\mu_{02(W_i\bar{Y}_{i\cdot})} + \bar{\bar{Y}}^2\,\mu_{20(W_i\bar{Y}_{i\cdot})}\right.$$

$$+ 2\bar{W}\bar{\bar{Y}}\mu_{11(W_i\bar{Y}_{i.})} \Big] + \frac{2}{n^2}\left[\bar{W}\mu_{12(W_i\bar{Y}_{i.})} + \bar{\bar{Y}}\mu_{21(W_i\bar{Y}_{i.})}\right]$$

$$+ \frac{1}{n^3}\left[\mu_{22(W_i\bar{Y}_{i.})} + (n-1)\left(2\mu^2_{11(W_i\bar{Y}_{i.})} + \mu_{20(W_i\bar{Y}_{i.})}\,\mu_{02(W_i\bar{Y}_{i.})}\right)\right]$$

$$- \frac{1}{n^2}\left[\mu_{11(W_i\bar{Y}_{i.})}\right]^2 .$$

Substituting (11), (13), (14) into (12), we obtain

$$(15)\quad MSE(\bar{y}') = \left(1 - \frac{2}{n}\right)\mu^2_{11(W_i\bar{Y}_{i.})} + \frac{1}{n}\left[\bar{W}^2\left(V + \mu_{02(W_i\bar{Y}_i)}\right) + \bar{\bar{Y}}^2\mu_{20(W_i\bar{Y}_{i.})} + 2\bar{W}\bar{\bar{Y}}\,\mu_{11(W_i\bar{Y}_{i.})}\right]$$

$$+ \frac{1}{n^2}\left[2\bar{W}\left(\mu_{12(W_i\bar{Y}_{i.})} + \mu_{11(W_iV_i)}\right) + 2\bar{\bar{Y}}\mu_{21(W_i\bar{Y}_{i.})} + V\mu_{20(W_i\bar{Y}_{i.})}\right]$$

$$+ \frac{1}{n^3}\left[\mu_{22(W_i\bar{Y}_{i.})} + \mu_{21(W_iV_i)} + (n-1)\left(\mu_{20(W_i\bar{Y}_{i.})}\,\mu_{02(W_i\bar{Y}_{i.})} + 2\mu^2_{11(W_i\bar{Y}_{i.})}\right)\right],$$

when N is large.

To compare the efficiency of the two estimators $\bar{y}$ and $\bar{y}'$ when N is large, (7) can be expressed as

$$(16)\quad V(\bar{y}) = \frac{1}{n}\left[\frac{1}{N}\sum_1^N (W_i\bar{Y}_i - \bar{Y})^2 + \frac{1}{N}\sum_1^N W_i^2 V_i\right],$$

where

$$(17)\quad \frac{1}{N}\sum_1^N (W_i\bar{Y}_{i.} - \bar{Y})^2 = \mu_{22(W_i\bar{Y}_{i.})} + 2\bar{W}\mu_{12(W_i\bar{Y}_{i.})} + 2\bar{\bar{Y}}\mu_{21(W_i\bar{Y}_{i.})} + \bar{W}^2\mu_{02(W_i\bar{Y}_{i.})} + \bar{\bar{Y}}^2\mu_{20(W_i\bar{Y}_{i.})} + 2\bar{W}\bar{\bar{Y}}\mu_{11(W_i\bar{Y}_{i.})} - \mu^2_{11(W_i\bar{Y}_{i.})};$$

$$(18)\quad \frac{1}{N}\sum_1^N W_i^2 V_i = \bar{W}^2\bar{V} + \bar{V}\mu_{20(W_i\bar{Y}_{i.})} + 2\bar{W}\mu_{11(W_iV_i)} + \mu_{21(W_iV_i)}$$

Hence

$$(19) \quad = MSE(\bar{y}') - V(\bar{y}) = \frac{n-1}{n} \mu^2_{11(W_i\bar{Y}_{i.})} - \frac{n-1}{n^2}\left[2\bar{W}(\mu_{12(W_i\bar{Y}_{i.})} + \mu_{11(W_iV_i)}) + 2\bar{\bar{Y}}\mu_{21(W_i\bar{Y}_{i.})} + V\mu_{20(W_i\bar{Y}_{i.})}\right] - \frac{(n-1)(n+1)}{n^3}\left[\mu_{22(W_i\bar{Y}_{i.})} + \mu_{21(W_iV_i)}\right] + \frac{n-1}{n^3}\left[\mu_{20(W_i\bar{Y}_{i.})}\mu_{02(W_i\bar{Y}_{i.})} + 2\mu^2_{11(W_i\bar{Y}_{i.})}\right].$$

Incidentally,

$$(20) \quad MSE(\bar{y}') = V(\bar{y}) + \Delta \quad .$$

In order to get the estimates of β and Δ, it is evident that the estimate of the former is

$$(21) \quad \hat{\beta} = \bar{y}' - \bar{y},$$

but the estimate of Δ will require the estimates of most of the parameters involved in that expression. The estimates of $\bar{W}$ and $\bar{\bar{Y}}$ are $\bar{w}$ and $\bar{\bar{y}}$ respectively. For the others, define

$$(22) \quad m_{rs(W_i'\bar{Y}_i)} = \frac{1}{n}\sum_{i}^{n}(W_i' - \bar{W})^r(\bar{y}_i - \bar{\bar{y}})^s.$$

Following the technique used in obtaining the $MSE(\bar{y}')$, we can prove, when N is large,

$$(23) \quad E(m_{20(W_i'\bar{y}_i)}) = \frac{n-1}{n}\mu_{20(W_i\bar{Y}_{i.})}$$

$$(24) \quad E(m_{02(W_i'\bar{y}_i)}) = \frac{n-1}{n}(\bar{V} + \mu_{02(W_i\bar{Y}_{i.})})$$

$$(25) \quad E(m_{11(W_i'\bar{y}_i)}) = \frac{n-1}{n}\mu_{11(W_i\bar{Y}_{i.})}$$

$$(26) \quad E(m_{12(W_i'\bar{y}_i)}) = \frac{(n-1)(n-2)}{n^2}\left[\mu_{12(W_i\bar{Y}_{i.})} + \mu_{11(W_iV_i)}\right]$$

$$(27)\quad E(m_{21(W_i'\bar{y}_i)}) = \frac{(n-1)(n-2)}{n^2}\ \mu_{21(W_i\bar{Y}_{i.})}$$

$$(28)\quad E(m_{22(W_i'\bar{y}_i)}) = \frac{n-1}{n}\ \frac{n^2-3n+3}{n^2}\left[\mu_{22(W_i\bar{Y}_{i.})} + \mu_{21(W_i V_i)}\right]$$

$$+ \frac{n-1}{n^2}\ \frac{2n-3}{n}\left[\mu_{20(W_i\bar{Y}_{i.})}\ \mu_{02(W_i\bar{Y}_{i.})} + 2\,\mu^2_{11(W_i\bar{Y}_{i.})}\right]$$

$$+ \left(\frac{n-1}{n}\right)^2 \bar{V}\ \mu_{20(W_i\bar{Y}_{i.})}$$

From these results, we get finally, after selecting terms involving $\frac{1}{n^3}$ and disregarding covariances between W_i', $m_{12(W_i'\bar{y}_i)}$; $\bar{\bar{y}}_i$, $m_{21(W_i'\bar{y}_i)}$; etc., the estimate of Δ,

$$(29)\quad \hat{\Delta} = \left(1 + \frac{6}{n^2}\right) m^2_{11(W_i'\bar{y}_i)} - \frac{2}{n-2}\left[\bar{w}\, m_{12(W_i'\bar{y}_i)} + \bar{\bar{y}}\, m_{21(W_i'\bar{y}_i)}\right]$$

$$+ \frac{3}{n^2}\ m_{20(W_i'\bar{y}_i)}\ m_{02(W_i'\bar{y}_i)} - \frac{1}{n}\left(1 + \frac{4}{n}\right) m_{22(W_i'\bar{y}_i)}$$

The calculation of $\hat{\Delta}$ is not prohibitive involving, fundamentally the items, $\bar{w}$, $\bar{\bar{y}}$, $W_i' - \bar{w}$, $\bar{y}_i - \bar{\bar{y}}$, and the sums of powers and products of powers of the last two.

The estimated relative efficiency of the estimator A to estimator B is given by

$$(30)\quad (R.E._{\hat{\bar{y}}|\bar{\bar{y}}}) = \frac{\hat{V}(\bar{\bar{y}}) + \hat{\Delta}}{\hat{V}(\bar{\bar{y}})} = 1 + \frac{\hat{\Delta}}{\hat{V}(\bar{\bar{y}})}$$

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A SEARCH FOR RESISTANCE TO THE INJURY CAUSED BY SPECIES OF DIABROTICA IN THE CORNS OF GUATEMALA[1]

Irving E. Melhus, Reginald H. Painter[2], and Frank O. Smith

Iowa State College-Guatemala Tropical Research Center
Antigua, Guatemala

Rootworm injury caused by species of Diabrotica was first observed on corn in Guatemala in 1945, 117 years after it was found by Yancy (1828) in the state of Illinois. The prevalence of the injury and its extent on different plants varied markedly. In some fields there was none; in others the roots were so badly injured that the plants were lying flat on the ground. Until it was possible to grow the corn strains in replicated plots in different altitude climates, with different dates of planting, and to carry out systematic examination of the roots, the rootworm injury picture was not clear.

In the tropics of Central America, rootworm injury is most severe on corn grown in climates between 2500 and 6500 feet altitude, designated as the highlands, and less so on the corn grown in the low coastal and high mountainous climates. In the highlands, rotation is not systematically practiced and the crop as a whole is seriously injured annually by species of Diabrotica.

That rootworm injury has prevailed for a long time in the highlands, although unknown to science, is apparent from the method of corn growing practiced by descendents of the Mayan people. High hilling to prevent lodging is as universally practiced as cleaning the fields of weeds. Where hilling is not practiced, much of the corn root-lodges. When this happens the damage to the crop often is serious. The lodged plants suffer poor pollination. The roots that may be healthy are broken and the photosynthetic process of the leaves is decreased incident to breaking and excessive shading. Damage to the crop varies but is often 50 per cent or more

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[2]Dr. Reginald H. Painter, Professor of Entomology of Kansas State College, was on a temporary summer appointment at the Tropical Research Center in 1951 and 1952. Contribution No. 625, Department of Entomology, Kansas State College.

in badly infested fields. Even when infested fields are hilled up high the damage is considerable because of the depleted root system. The plants dry up and the grain is light and shriveled. Certainly the rootworm causes serious losses annually to the corn crop of the Guatemalan highlands.

In Guatemala it is safe to assume that corn has been a host of the larval stage of some species of Diabrotica ever since the Mayan people settled in the highlands in the pre-Columbian period. In other words, this insect pest and the corn of the highlands may well have been associated for 5000 years or more. During this period, extensive natural and artificial selection has taken place in the corn plant, giving rise to many strains and varieties differing in earliness, size, color, productivity, disease resistance, adaptability to altitude and latitude climates, etc. Similarly, selection may well have taken place in the reaction of different corns in this region to injury caused by species of Diabrotica. This fact, coupled with the annual prevalence and destructiveness of Diabrotica larvae, suggested that the highlands might be a good place to search for rootworm resistant strains of corn. Such a search has been carried on during the last seven years, 1946 to 1952 inclusive, by the senior author.

The annual prevalence and destructiveness of species of Diabrotica is probably facilitated by the mildness of the climate and the corn growing practices. There are two seasons in Guatemala, a wet and a dry, generally of about equal length. It is during the wet season that the main corn crop is grown. During this period the rainfall is high, varying in different regions from 20 to more than 150 inches in a six- or seven-month period. Throughout the dry season the temperature is favorable for species of Diabrotica, so the insect population is not reduced as much as in more northern latitude climates where the ground freezes for periods of from three to six months, as in the corn belt of the United States. This favorable climate for species of Diabrotica and the growing corn crop ensures the annual occurrence of much damage by Diabrotica species. Thus, the Guatemalan highlands are an ideal place to study rootworm injury, much more so than the United States where rootworm damage occurs in some seasons and not in others.

The rootworm injury in Guatemala is much like that caused by the southern corn rootworm, Diabrotica duodecimpunctata F., now known as D. undecimpunctata howardi Baker, and by other species in the United States as described by Chittenden (1910), Marsh (1910), Webster (1913), Forbes (1896), Isley (1929), Tate and Bare (1946), and others. However, in Guatemala injury is caused by species other than D. duodecimpunctata.

TIME OF PLANTING IN RELATION TO ROOTWORM INJURY

Field observations indicated that the amount of rootworm injury varied with the time of planting. The indigenous people have fixed dates for planting their corn; these vary from one climatic zone to another. For example, in the Antigua region, 3000 to 6500 feet, most of the crop is planted in late February and during all of March. On the coast, from sea level to 2000 feet, the crop is planted just preceding or following the first rain of the rainy season. In the low Pacific coastal area where the mean temperature ranges from 10 to 12 degrees higher than in the highlands, the rootworm injury is not common and seldom causes any serious

damage. Undoubtedly these practices have evolved slowly over a period of many years as a result of observation of the effect without knowledge or understanding of the cause. Experimental evidence presented later substantiates the validity of the practice of early planting.

DIABROTICA SPECIES ON CORN

There are many species of Diabrotica that occur on corn in Guatemala. They occur throughout the year in the adult stage on the first and second crops. In the highlands where there is only one corn crop a year, the beetles feed on other crops such as squash, beans, alfalfa, and on broad-leaved weeds. When volunteer corn is present, they feed on the corn. As soon as the crop planted in March and April reaches eight to ten inches in height, beetles appear, probably migrating from other host plants.

Adults of more than a dozen Chrysomelidae[1] have been taken on corn, feeding on tender leaves, silks or tassels in Guatemala. The following annotated list, giving collection localities and hosts of adults, is arranged approximately in order of the abundance on the trial grounds at Antigua.

1. Diabrotica adelpha Harold. Antigua (corn, beans, teosinte, alfalfa); Coban (corn, squash); Barcena (corn).
2. Diabrotica balteata LeConte. Antigua (corn, beans, teosinte); Barcena (corn); Tiquisate (corn).
3. Diabrotica porracea Harold. Antigua (corn, teosinte); Barcena (corn); Coban (squash); San Jose Pinula (corn); S. Maria de Jesus (corn).
4. Diabrotica sp. near porracea. Antigua (corn, teosinte); San Jose Pinula (corn).
5. Diabrotica longicornis new subspecies (= viridula auct. nec Fab. in part). Antigua (corn, beans); Barcena (corn); Coban (corn).
6. Acalymma trivittata (Mann.). Antigua (corn).
7. Diabrotica sp. near bioculata Bowd. Antigua (corn, beans, alfalfa).
8. Andrector atrofasciatus (Jacoby). Antigua (corn, beans, teosinte). Coban (most common on beans).
9. Diabrotica rufomaculata Jacoby. Antigua (corn).
10. Diabrotica sexmaculata Baly. Antigua (corn, beans).
11. Diabrotica nigrofasciata Jacoby. Antigua (corn, beans; most common on beans).
12. Diabrotica tibialis Jacoby. Antigua (corn).
13. Diabrotica tricolor Jacoby. Antigua (corn).
14. Diabrotica lepida (Say). Antigua (corn).
15. Amphelasma cavum cavum (Say). Antigua (corn).
16. Acalymma cornuta (Baly). Antigua (corn).
17. Monolepta sp. Antigua (corn); Barcena (corn).

Diabrotica undecimpunctata howardi Baker (D. duodecimpunctata F.) has not been collected during these investigations. D. balteata was the only species of this group seen on the Pacific coastal plain; D. porracea was the last species to disappear in the corn fields above about 6500 feet.

Diabrotica larvae are most abundant on the roots in June and July of the crop planted in April. The larvae are not prevalent on the corn roots

[1]The authors are indebted to the staff of the Division of Insect Identification of the Bureau of Entomology and Plant Quarantine U.S.D.A., and to Ray F. Smith of the University of California, for these identifications.

during the dry season. This may be the result of the lack of sufficient moisture for the eggs to hatch and the larvae to reach the young permanent roots. In June, the small white larvae are often abundant in the surface inch of soil about the stem of the plant. In late June and July the tunnelled, permanent roots are common and the larvae can readily be found feeding in the tissues and in the soil about the permanent roots. The crown and seminal roots were almost always free from root injury in the early part of the season. Later on, when the infestation was heavy, the crown and seminal roots might also be attacked. By the middle of July, part or all of the permanent roots were killed and the plants root-lodged unless hilled very high with soil, a practice that is followed regularly by the Indians in the highlands where rootworm injury occurs.

Rootworm injury seldom becomes pronounced on corn roots until the permanent roots appear. This is true even when corn is planted during the rainy season when conditions seem to be optimum for the female to lay eggs and the larvae to feed on the corn roots.

SOME SPECIES CAUSING ROOTWORM INJURY

In 1952 an attempt was made to learn what species of Diabrotica cause rootworm injury. From August 10 to 15, twenty-eight hills of corn were caged. The cages were of wood, about one foot square and one foot tall. The tops of the cages were covered with a fine mesh lumite screen. The corn plants were cut just above the crown, the few weeds present were removed, and the wood frame was sunk about two inches into the soil. The kernels of the plants were in the milk stage between 110 and 130 days after planting. The caged corn plants were all inbreds of 92A-46, 1483-45 5s, and 20-47 2s. Rootworm injury was present on all the caged plants when dug after the cages were removed 30 to 36 days after they were caged.

TABLE 1

Dates of Emergence of 15 Diabrotica from Cages Over Corn Stubs, Antigua, Guatemala, August 1952

	Species of Diabrotica					
Date	adelpha	balteata	porracea	sp. near porracea	sp. near bioculata	rufomaculata
8/15	1 Cage 14		1 Cage 14			
8/19	1 Cage 5	1 Cage 5	1 Cage 1	1 Cage 15*		1 Cage 19*
	1 Cage 14	1 Cage 27	1 Cage 7*			
8/20		1 Cage 5				
8/22	1 Cage 1					
8/30				1 Cage 5	2 Cage 5	

* General specimens; identifications questionable.

A total of 15 adult Diabrotica were removed from the cages on the dates indicated in Table 1. None of these beetles were taken after August 31, 1952 and the experiment was discontinued September 15. Hence only the end of the emergence of the adults was recorded. Various other insects were removed from the cages including twelve small wireworm adults, Horistonotus refiventris Cand. The larvae of the latter may have been responsible for some damage to the corn roots but the larvae of Diabrotica species have been the ones commonly found feeding.

In the field and in the laboratory, adults of D. balteata, D. porracea and D. adelpha fed readily on leaves of seedling corn. Eggs were secured from these three in the laboratory. Larvae hatched from eggs of D. adelpha and gave typical injury to roots of corn seedlings, eventually killing the plants. These larvae failed to establish themselves on the new seedlings to which they were transferred.

In another experiment, three species of Diabrotica were caged on young corn plants. Six cages were planted to corn and seedlings transplanted from the laboratory on August 15. Adult Diabrotica beetles were placed in them on August 21 when the transplants were 6 to 8 inches tall. Two cages each contained D. balteata, D. porracea and D. adelpha. The cages were kept sealed with gummed paper so that there was little probability that insects entered the cages above ground. The cages rested on ground that had not been in corn for five years, precluding the presence of larvae in the soil, although some migration of the larvae from cage to cage, through the ground, may have occurred.

On October 4, two plants were lifted from each of the three cages containing the three species. The plants in each of three cages showed some rootworm injury where the permanent roots had developed. There was no injury where the permanent roots had not developed, although larvae were present in the soil in each case. The seminal roots were not attacked. There were more larvae in the D. balteata cage than in the other two. The roots of the plants exposed to D. porracea, showed more injury than the other two. One plant had 3 and the other 1 class of injury. (See classes of injury in Fig. 1.) The injury caused by D. balteata and D. adelpha was 1. The small amount of injury was doubtless due in part to the absence or very recent development of the permanent roots.

Another record of the root injury in this experiment was taken November 19, or 90 days after seeding of the cages. The results obtained were as follows:-

Species	No. of plants	No. of root whorls	Class of injury
D. porracea	1	4	4
	3	3-4	2
	1	3	3
	2	3-4	1
D. adelpha	2	4-5	2
	1	4	1
	1	4	3
D. balteata	4	3-5	3
	1	3	2
	1	3	1

Fig. 1. Different classes of rootworm injury to corn roots. These root systems illustrate the different numerical classes used in evaluating the injury on different strains and varieties studied. The numerical classes are:

1. Trace to 10 per cent of roots destroyed
2. 11 " 30 " " " " "
3. 31 " 50 " " " " "
4. 51 " 70 " " " " "
5. 71 "100 " " " " "

Fig. 2. The injury caused on corn plants 90 days after they were caged. Three species of Diabrotica were used, namely; D. balteata, D. porracea and (unlabeled) D. adelpha. The larvae of each species fed on the crown roots, causing typical rootworm injury. Antigua, Guatemala, 1952.

Four of the plants were killed by high moisture conditions inside the cages. There were also some dark lesions not caused by insects on the stems at or just above the crowns of the plants. Some of the crown and seminal roots were discolored and soft incident to soft rot bacteria. However, the other plants developed well considering the confined condition. The root development was better than that of the aerial parts of the plants. There was considerable variation in the number of root whorls, due to the different ages of the plants, but this seemed to have little relation to the amount of injury. Each species produced larvae that fed on the permanent roots. The amount of injury varied from 1 to 4, as shown above and in Fig. 2. The data are too limited to indicate which species fed most extensively on the roots but it was clear that the plants caged with D. balteata, D. adelpha, and D. porracea showed characteristic rootworm injury, like that occurring in the experimental plots.

THE DEVELOPMENT OF PERMANENT ROOTS IN RELATION TO ROOTWORM INJURY

A study was made of the development of the root systems in relation to rootworm injury on 15 entries shown in Table 2. Thirteen Guatemalan varieties and two U.S. (one single cross and one inbred were used). The 13 guatemalan varieties were chosen because in previous trials they had shown a wide range of susceptibility. The inbred 38-11 was chosen because of its reputed resistance to Diabrotica duodecimpunctata, as reported by Huber, Seem, Coon and Wernham (1948), Bigger, Snelling and Blanchard (1941), and others. These corns were planted on April 15 in eight-foot replicated rows, 50 kernels per row. There was sufficient soil moisture below the dust mulch to promote good growth. The time of planting was during the dry season, and no rain fell during the first 33 days of their growth. On the 34th day, a half inch of rain fell making available adequate moisture for the plant to continue good growth.

In order to follow the development of the root system and to observe the rootworm injury, ten plants were dug up from each entry on three different dates, 35, 55, and 86 days after planting. No permanent roots had developed during the first 35 days. There was no rootworm injury. After 55 days, from 2 to 3 permanent root whorls had developed. There was no appreciable difference in the number of permanent root whorls in the early and late corns. The two U.S. corns had the same number of root whorls as the Guatemalan corns. This was true of 26A-46 # ⊗, an early Guatemalan variety, as well as the other Guatemalan varieties which were either intermediate or late. Again there was no visible rootworm injury. On the 86th day, the number of crown root whorls[1] varied from 3 to 4 and in most cases one whorl was above the soil. Rootworm injury was in evidence as indicated in Table 2. Small white larvae were taken in the roots and soil. These resembled the larvae of species of Diabrotica.

The data suggested that there was a lapse of some time after the permanent roots developed before rootworm injury appeared. There was no

[1] Crown root whorls, as used, designate the whorls of adventitious roots at and above the distal end of the mesocotyl.

TABLE 2

The Growth Response and Development of Rootworm Injury on Thirteen Guatemalan Varieties and Two U.S. Corns, One Single Cross and One Inbred Line, Antigua, 1950

Entry no.	Pedigree	Development after 35 days: Height inches	35 days: Diameter inches	35 days: Mesocotyl length inches	35 days: Mean no. seminal roots	55 days: Height inches	55 days: Diameter inches	55 days: Root whorls	55 days: Mean no. crown roots	86 days: Height inches	86 days: Diameter inches	86 days: Root whorls	Mean class injury 10 plants
1	89-49	23	0.2	1.5	3	45	0.5	2	11	48	0.75	3	5.0
2	Wf9 x 38-11	20	0.2	0.75	2	40	0.5	2	10	60	0.5	4	5.0
3	Ind. 38-11	20	0.2	0.75	4	35	0.5	2	14	48	0.5	3	4.6
4	192-44 2s	21	0.2	0.87	3	40	0.5	2	12	72	1.0	4	2.6
5	89A-46#	13	0.2	0.87	3	45	0.5	2	10	60	1.0	4	3.0
6	1570-45#⊗	20	0.75	1.25	2	45	0.5	2	14	60	0.75	4	5.0
7	1483-45#⊗	19	0.42	0.87	5	40	0.5	2	10	72	0.75	4	3.2
8	26A-46#⊗	16	0.42	1.00	3	48	0.5	3	12	60	0.5	4	4.7
9	1626-45#⊗	16	0.42	1.00	5	40	0.5	2	15	72	0.75	4	4.4
10	35y 2s	17	0.2	1.12	3	38	0.5	3	10	72	0.75	4	3.2
11	92A-46⊗	16	0.31	1.25	5	40	0.5	2	14	48	0.5	4	2.5
12	47A-46	24	0.42	1.00	5	45	0.5	2	11	48	0.75	4	4.2
13	1609-45#	24	0.42	1.00	4	45	0.5	2	12	--	--	-	-
14	1555-45#	12	0.2	1.12	1	40	0.37	2	12	--	--	-	-
15	1471-45#	28	0.56	2.25	4	55	0.37	3	11	60	0.75	4	3.4

Entry No. 2 was a U.S. single cross and No. 3 was a U.S. inbred line. All the other entries were Guatemalan corns.

rootworm injury after 55 days, although 2 to 3 permanent root whorls had developed. Under the conditions of the experiment, the depositing of the eggs by species of Diabrotica and the development of the larvae occurred between the 35th and 86th days.

It is significant that the time of permanent root development in 15 different varieties occurs at about the same time, on early and late varieties, which suggests that relative susceptibility can not be correlated with difference in time of permanent root appearance. Injury to the root systems occurred only after the soil became wet. There was a lapse of about 30 days from the time the permanent roots began to develop until serious injury occurred to the root tissues. The permanent or crown roots were preferred by the larvae to the seminal roots.

The reaction of the resistant strains, 192-44 2s and 92A-46 ⊗, was not incident to the tardy development of permanent roots because they had as many root whorls as the earlier maturing corns, U.S. inbred 38-11, the single cross Wf9 x 38-11, and the early Guatemalan variety 26A-46. This was true also of the Guatemalan varieties of intermediate maturity namely, 1471-45 and 1626-45.

ROOTWORM INJURY ON CORN IN THE DRY SEASON

Corn, started in the dry season, escapes much rootworm injury in the Antigua region and probably also in other parts of the highlands. This may be responsible for the practice of planting in February and March in many localities in the highlands. The Indian has no explanation for this practice other than that the crop is likely to be better and that it is the custom. The fact that February and March plantings produce better crops than plantings in April, May and June has been well established. The increased yield is probably not only the result of less rootworm injury, but also of length of day reaction and other causes. However, there can be no doubt that the absence of serious rootworm injury is an important consideration in the increased yields incident to early planting.

To shed light on the amount of rootworm injury on February and March plantings, 11 Guatemalan varieties and 4 U.S. hybrids were planted in 60 hill replicated rows on the Antigua trial grounds in 1950. The plantings were made February 15, and March 16 and 21. The Guatemalan varieties used included three varieties that in previous trials had shown considerable resistance, namely, 35y, 92A-46 and 192-44, and one variety that had proved very susceptible, 100A-46. The other seven were intermediate in rootworm injury reaction.

The land used had grown a crop of corn the previous year. Before that it was in poor pasture consisting of grasses and weeds. The land was plowed in November 1949 just at the close of the rainy season and disced a few days later to develop a loose soil mulch to conserve the soil moisture. Germination and growth of the corn were good except for some damage from maize maggot (Euxesta major v.d. Wilb). There was no rain until May 19 (one-half inch), the beginning of the rainy season in Antigua. In other words, the February 15 planting received no rain during the first 92 days of its growth and the March 16 and 21 plantings, 64 and 59 days, respectively. On June 6, 118 days after the first planting, ten hills were dug from each entry and the amount of rootworm injury evalu-

ated according to an established scale (Fig. 1). The mean class injury is shown in Table 3. There was little rootworm injury, although Diabrotica species were prevalent on the growing corn, feeding on the tassels and silks (Fig. 3). This was definitely a case of escape because the four U.S. hybrids known to be very susceptible, showed little injury; the same was true of the Guatemalan susceptible and resistant varieties. The injury that did occur was largely tunnelling and root pruning of the permanent roots. The seminal roots were clean and there naturally was no lodging because the plants had a strong complement of permanent roots. This experimental evidence and earlier observations indicated clearly that in any search for resistant varieties the plantings should be made later than March 21, in order to ensure severe rootworm injury.

TABLE 3

Rootworm Injury on Eleven Different Guatemalan and Four U.S. Hybrids Grown Under Dry Season Conditions

Date planted	Pedigree		No. of root whorls	Class root injury 0	1	2	3	4	5	Mean class injury
Feb. 15	1570-45	Guate.	4-5		5					1.0
	92A-46	Guate.	3-7		5					1.0
	35y ⊗	Guate.	4-6		4	1				1.2
	MS 59	U.S.	3-4	3	7					0.7
	Wf9 x 38-11	U.S.	3-4	3	7					0.7
	67-44#	Guate.	2-3	4	5	1				0.6
	100A-46 ⊗	Guate.	3-4			5	5			2.5
	20-47 ⊗	Guate.	3		7	1				0.9
	92A-46 #	Guate.	3-4	2	8					0.8
	MS 99	U.S.	3-4	1	6	2	1			1.2
	192-44	Guate.	4-6		4	1				1.2
									Av.	1.1
Mar. 16	MS 99	U.S.	3-4		6	3	1			1.5
	129A-46#	Guate.	3		5	4	1			1.6
	100A-46 #	Guate.	3		3	2	3	2		2.4
	187-2 x L317	U.S.	3		3	6	3			2.4
	20-47	Guate.	3		6	2	2			1.6
	MS 59	U.S.	4	2	6	2				1.0
	92A-46 2s	Guate.	2			5				2.0
	102A-46 2s	Guate.	2-3			5				2.0
	192-44 2s	Guate.	2			5				2.0
									Av.	1.8
Mar. 21	1555-45	Guate.	2			4	1			2.2
	12A-46 ⊗#	Guate.	2-3			4	1			2.2
	92A-46	Guate.	3-4		9	1				2.2
	102A-46	Guate.	3-4		3	3	3	1		2.2
	192-44	Guate.	3-4		4	3	3			1.9
	1555-45	Guate.	2-3		3	3	2	2		2.3
									Av.	2.2

THE SEARCH FOR RESISTANT STRAINS AND VARIETIES DURING 1949 TO 1952 INCLUSIVE

All the strains and varieties that have been grown and screened for rootworm injury were planted in late March and the first half of April on part of the Antigua trial grounds that had been in corn the previous year. The land used was of volcanic origin. The soil was nearly neutral in reaction, ranging from pH 6.8 to 7.2. It was low in organic matter, water holding capacity, and fertility. Side dressings of ammonium sulfate or sodium nitrate were used in 1951 and 1952. The land was plowed each year just at the close of the wet season. All refuse on the plots was plowed under. After plowing, the land was disced if lumpy so as to ensure a loose surface mulch. The soil was not disturbed again until planting time the next year. The planting was all done with a hoe by moving away the dry mulch and dropping the seed into the moist soil. Three or four kernels were planted in each hill, and the hills were spaced 3 x 3 or 3.5 x 3.5 feet. In each experiment the entries were planted in two or three randomized blocks. No cultivation was practiced until about a month after the rainy season started. Each year some cucurbit (cucumbers or squash) was planted in limited vacant areas on the plots. Beans, both edible and non-edible, were always present in adjoining plots.

The Diabrotica population developed spontaneously and was always abundant on the growing corn. Larvae of Diabrotica species were always prevalent in the soil about the permanent roots in June and July, but less so in August and September. The temperature and rainfall for the growing season of each year are shown in Table 4.

TABLE 4

Temperature and Rainfall at Finca Retana, Adjoining the Antigua Trial Grounds 1949 to 1952 Inclusive

Month and Year	Temperature C.			Rainfall		Month and Year	Temperature C.			Rainfall	
	Max.	Min.	Mean	Days	Inches		Max.	Min.	Mean	Days	Inches
March 1949	25	7	16.0	—	—	March 1950	25	8	16.5	1	.08
April	26	11	18.5	2	.30	April	26	7	16.5	—	—
May	25	11	18.0	11	4.67	May	26	12	19.-	14	4.33
June	23	12	17.5	22	6.40	June	24	12	18.-	19	8.0
July	24	11	17.5	8	1.94	July	25	9	17.-	63	16.16
August	24	10	17.0	17	6.80	August	25	10	17.5	17	3.6
September	22	12	17.0	24	12.57	September	24	13	18.5	22	8.91
March 1951	27	6	16.5	—	—	March 1952	27	4	15.5	3	.77
April	26	8	17.0	1	.04	April	29	8	18.5	6	2.06
May	25	8	16.5	13	4.81	May	25	12	18.5	17	4.41
June	26	11	18.5	14	3.45	June	26	11	18.5	24	10.15
July	25	12	18.5	17	6.68	July	26	11	18.5	21	7.10
August	26	11	18.5	11	2.69	August	26	11	18.5	17	3.82
September	24	10	17.0	26	14.60	September	24	11	17.5	24	5.93

The mean temperatures for May, June and July for the four years were quite uniform, except in May of 1951 when the temperature was about two degrees lower than in the other three years. The rainfall in March and April of 1949, 1950, and 1951 was only 0.42 inch. In 1952, on the other hand, 2.83 inches fell on nine different days. The rainy season began abnormally early in 1952. The rainfall of May and June of each year was favorable for the development of the larval stage of Diabrotica species.

Variability in Resistance of Strains and Varieties to Rootworm Injury

Observations and field trials in 1946 and 1947 indicated that not all strains and varieties of corn in the highlands were equally injured by species of Diabrotica. Whether plant resistance or merely escape were involved was not apparent. Little work has been done on rootworm injury; however, there is some evidence that resistance to the Southern corn rootworm does exist in the United States. Bigger, Holbert, Flint, and Lang (1938) and Bigger, Snelling, and Blanchard (1941) found a differential response to the destructive feeding of the larvae and the Indiana inbred line, 38-11, to be outstanding in its resistance to lodging. Lodging was used as an index of resistance. Huber, Seem, Coon, and Wernham (1948) also rated Indiana 38-11 high in root quality. Painter (1951) recently summarized the data on rootworm resistance previous to 1950. In 1951 Melhus reported a search for resistance for the previous four years in Guatemala and stated that strains had been isolated showing comparatively little root injury.

The rootworm problem in the United States is different than that in Guatemala because the Diabrotica species involved, in part, in the two countries are different.

In order to learn more about the possibility of finding strains and varieties resistant to rootworm, about 600 corn collections were grown during 1947 to 1950 inclusive. One of the factors that sharply influenced the amount of rootworm injury was that of time of planting, discussed earlier. In 1949 and 1950 the rootworm trials were all planted between March 31 and May 12, planting dates that had yielded heavy rootworm injury in previous years.

Three hundred and ten lines and varieties, largely collected in Guatemala, were grown and rated for rootworm injury in 1949 and 1950 on the Antigua trial grounds. These strains and varieties all fell in the four groups described by Melhus, Wallin, and Semeniuk (1949). The different trials were planted in three randomized blocks, ten hills per entry. Four seeds were planted per hill and the hills were spaced 3 x 3 or 3.5 x 3.5 feet apart. The rootworm records were taken by digging the plants in September and recording the injury class, using the scale shown in Fig. 1. The records each year were taken by the same two assistants, supervised by the senior author.

The extent of the rootworm injury varied from one year to another even though the crop was planted about the same time each year. Thus, the class injury distribution differs from one season to another (Figs. 3-6). For example, if the amount of rootworm injury was low, the number of strains or varieties in the low classes increased, as in the 107 entries shown in Fig. 4. There were nine entries with less than 2.5 injury, while

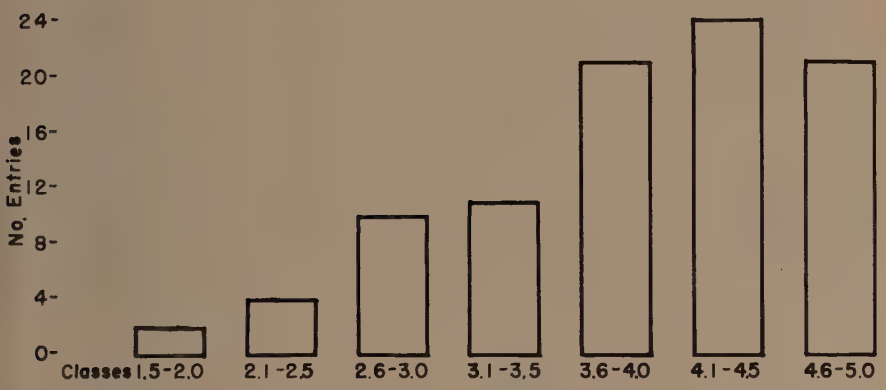

Fig. 3. This graph shows the response of 93 different inbred lines of varieties, grown in 1950. The plantings were made March 29. There were 4.32 inches of rain during the last half of May. Two lines 1555-45 3s and 47-44, inbred three generations, fell in the lowest group of injury. Four lines, also inbred three generations, fell into the 2.1 to 2.5 group.

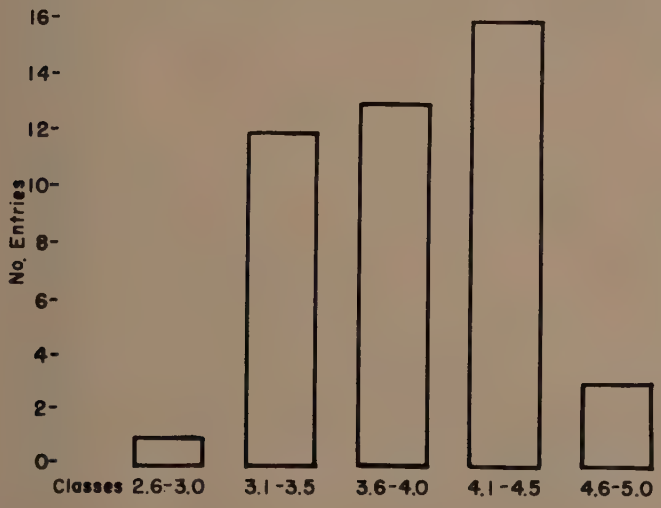

Fig. 4. This graph shows the response of 47 entries planted on April 10, 1950. The varieties were open pollinated or had been selfed once. The injury was read 149 days after planting. Only one variety, 1651-45, showed a 2.8 class injury.

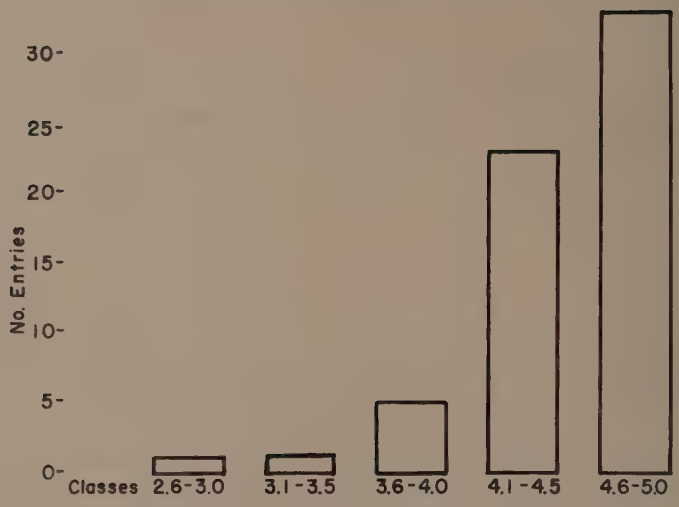

Fig. 5. In 1949, 63 varieties were planted April 4 previous to the beginning of the rainy season. The rainfall the last half of May was 4.67 inches. The varieties in trial were either open pollinated or selfed lines. All of the varieties except two, showed class injury in the range 3.6 to 5.0. These two lines were 192-44 ⊗ and 81-44 # ⊗, lines belonging to the Giganteum group.

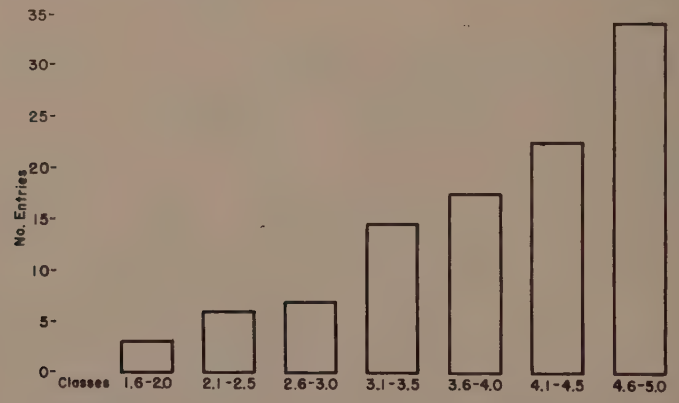

Fig. 6. One hundred and seven entries were planted March 29, 1950. These were all varieties selfed twice. There was no rain in April, but 4.33 inches fell the last of May, six to seven weeks after planting. The comparative root injury was taken immediately after the crop was harvested in September. Nine entries were in the two groups, 1.6 to 2.0 and 2.1 to 2.5. These were lines of the varieties 21-47, 105A-46, 146-47, 182-47, 284, 1609-45, 1652-45, 47-44, and 25A-46.

in 1949 only two entries fell in the class groups 2.6 to 3.0 and 3.1 to 3.5. Obviously there were factors other than time of planting that influenced the amount of rootworm injury, such as the degree of favorable environment for the beetles, varieties in test, population of beetles, etc. However, it is clear that most strains and varieties were highly susceptible and that the most resistant ones belonged in the Giganteum group, rather than in the Coast, Early or Mountain groups. All eighteen strains or varieties cited in the legends of Figs. 3 to 6 belonged to the Giganteum group with the exception of 12A-46, which belonged to the Coast group. It is quite conceivable that several of the eighteen strains may, in subsequent tests, fall in higher or lower class groups within certain limits.

The segregation of eighteen strains and varieties from the 310 in trial afforded an opportunity to choose strains or varieties for further, detailed study of their resistance reactions.

Resistance in the Second Generation Selfs of Twenty-four Lines of 92A

To determine whether resistance in strains and varieties really exist, nine of the 18 entries were inbred from one to four generations and tested again. The response of one of these open pollinated varieties, 92A, will serve to illustrate the response obtained. Twenty-four lines chosen at random from more than 300 second generation selfs of 92A, were planted in randomized, replicated plots in 1951 and 1952. A variety known from previous trials to be susceptible, namely 1570-45 # ⓧ was included as a check. The mean class root injury is shown in Table 5. Each entry consisted of ten hills in each replication. Ten plants were dug from each entry in each replication and the rootworm injury recorded. There was more rootworm injury in 1951 than in 1952. The mean class injury of the 24 lines in 1951 and 1952 was 3.05 and 2.59 or a difference of 0.46. The

TABLE 5

The Comparative Response of Twenty-four Lines of 92A Inbred Two Generations and a Check in 1951 and 1952

Pedigree	Mean class root injury 1951	1952	Pedigree	Mean class root injury 1951	1952
92A 2s ear -3	3.4	2.7	92A 2s ear -17	3.3	4.0
" " " -4	4.2	2.5	" " " -20	1.5	2.2
" " " -5	3.2	2.2	" " " -22	3.8	2.0
" " " -6	3.5	2.4	" " " -23	3.7	2.6
" " " -7	3.4	2.1	" " " -25	2.2	2.4
" " " -9	3.4	2.0	" " " -26	3.5	3.1
" " " -10	3.0	3.7	" " " -27	2.4	3.3
" " " -11	3.6	3.4	" " " -28	2.6	2.5
" " " -12	3.9	2.3	" " " -29	1.9	1.9
" " " -13	3.4	2.2	" " " -32	3.1	2.7
" " " -14	1.5	2.8	" " " -43	3.2	1.6
" " " -16	2.7	2.0	" " " -75	2.9	3.7
			1570-45#ⓧ	4.6	4.5

range of variation in 1951 was 1.5 to 4.2 and in 1952, 1.6 to 4.0. The mean class injury of each of the 24 lines was less than the susceptible check. Also the data show that inbreds of an open pollinated resistant population comprise lines that are resistant.

The Response of Thirty-nine Lines of 92A Inbred Three Generations in 1951 and 1952

To determine still further whether resistance exists in Guatemalan corns, 39 third generation inbreds of 92A were grown in three randomized blocks, ten hills per entry along with a check 1570-45#⊗. These 39 inbreds showed some loss of vigor incident to inbreeding in that they were not so tall and the ear shoots were smaller. There was no attempt made to select lines that in the previous generation showed high resistance. They were taken at random from the selfed ears grown the previous year. As in the previous trial, with second generation lines, the third generation lines showed more root injury in 1951 than in 1952 (Table 6). However, all the lines showed less injury than the check. The loss of some vigor of the third generation lines seemed to have little effect on the degree of resistance. The resistance manifested in the second generation, was present in the third, definitely indicating that resistance exists in Guatemalan corns and that it is heritable. The degree of resistance in one of the best lines, 92A 3s ear 58, is shown in Fig. 7.

TABLE 6
The Response of Thirty-nine Lines of 92A Inbred Three Generations

Pedigree	Mean class root injury 1951	1952	Pedigree	Mean class root injury 1951	1952
92A 3s ear -31	2.4	3.2	92A 3s ear -60	2.4	2.1
" " " -33	3.3	3.2	" " " -61	3.0	1.9
" " " -34	3.8	3.1	" " " -62	3.7	2.5
" " " -35	4.0	2.0	" " " -63	2.2	2.8
" " " -39	3.5	2.3	" " " -64	3.9	2.9
" " " -40	3.6	2.8	" " " -66	3.0	2.3
" " " -41	3.9	2.0	" " " -68	1.9	2.6
" " " -42	3.9	2.7	" " " -69	3.4	2.7
" " " -44	3.7	1.7	" " " -70	3.3	2.5
" " " -45	3.5	2.4	" " " -71	3.7	2.5
" " " -47	3.2	3.3	" " " -73	2.4	2.1
" " " -49	3.2	2.7	" " " -74	3.2	3.5
" " " -50	3.3	3.1	" " " -76	3.7	3.5
" " " -51	3.3	3.1	" " " -77	4.0	2.0
" " " -52	2.7	2.1	" " " -78	2.9	3.3
" " " -54	3.2	2.5	" " " -80	2.8	1.7
" " " -55	2.8	2.4	" " " -81	3.5	3.0
" " " -56	3.5	3.6	" " " -83	3.5	3.0
" " " -58	2.0	1.6	" " " -84	3.3	2.7
" " " -59	3.2	3.0	1570-45#⊗	4.6	4.5

Fig. 7. The progeny of 92A 3s ear 58 showed a 2 class injury in 1951 and a 1.6 in 1952. Through the selection of such inbred lines it is possible to fix resistance above the mean of a collection of lines chosen from an open pollinated population.

The Response of 92A Crossed on (187-2 x L 317)

In order to learn whether resistance in 92A was transmitted to hybrids, it was crossed on a susceptible U.S. single cross (187-2 x L 317). The inbreds of 92A were in the first and second generation. Fifty-two hybrids and the two parents were planted in two randomized blocks. Each entry consisted of 10 hills in each block. The seed was planted April 9, 1951, on the Antigua trial grounds. Thirty plants per entry were dug and examined for root injury in early September after the plot was harvested. The U.S. single cross (187-2 x L 317) showed a 4.0 class injury, and 92A, a 2.9. The range of injury of the 52 three-way hybrids was 1.9 to 3.6. The mean of the 52 hybrids was 2.76. The data in Table 7 indicate that inbreds of 92A in hybrid combination with a susceptible U.S. single cross were all more resistant than the susceptible parent and more than half of the 52 hybrids were more resistant than 92A o.p. The comparative resistance of four hybrids and the susceptible check are shown in Fig. 8. Resistance was inherent in 92A and was transmitted to some of the hybrids.

TABLE 7

The Response of Rootworm Injury of Fifty-two Hybrids and Two Checks in 1951

Pedigree				Mean class root injury		Pedigree				Mean class root injury	
(187-2 x L 317)	92A 2s	ear		-1	3.5	(187-2 x L 317)	92A ⊗	ear		-43	3.4
"	"	"	"	-3	2.9	"	"	"	"	-44	2.6
"	"	"	"	-5	2.6	"	"	"	"	-45	3.0
"	"	92A ⊗	"	-8	2.1	"	"	"	"	-46	3.1
"	"	92A 2s	"	-8	2.8	"	"	"	"	-48	2.7
"	"	"	"	-9	2.3	"	"	"	"	-49	3.1
"	"	"	"	-10	2.7	"	"	"	"	-50	2.7
"	"	"	"	-11	2.9	"	"	"	"	-51	2.5
"	"	"	"	-12	2.7	"	"	"	"	-53	3.1
"	"	"	"	-14	2.6	"	"	"	"	-54	2.4
"	"	"	"	-15	2.4	"	"	"	"	-56	3.2
"	"	92A ⊗	"	-18	2.3	"	"	"	"	-57	3.3
"	"	"	"	-19	2.7	"	"	"	"	-58	2.4
"	"	"	"	-20	2.5	"	"	"	"	-59	2.6
"	"	"	"	-21	3.1	"	"	"	"	-61	3.1
"	"	"	"	-24	3.2	"	"	"	"	-62	2.3
"	"	"	"	-25	2.5	"	"	"	"	-64	3.6
"	"	"	"	-26	2.5	"	"	"	"	-64	1.9
"	"	"	"	-28	2.4	"	"	"	"	-65	2.1
"	"	"	"	-29	2.9	"	"	"	"	-66	2.3
"	"	92A 2s	"	-30	3.3	"	"	"	"	-69	3.2
"	"	92A ⊗	"	-32	2.8	"	"	"	"	-70	3.3
"	"	92A 2s	"	-33	2.9	"	"	92A 2s	"	-71	3.0
"	"	"	"	-34	2.4	"	"	"	"	-73	2.4
"	"	"	"	-40	2.9	"	"	"	"	-76	3.1
"	"	"	"	-41	2.6	U.S. 187-2 x L 317					4.0
"	"	"	"	-42	2.5	92A o.p.					2.9

SUMMARY

The annual prevalence and destructiveness of species of Diabrotica are favored by the climate, soil conditions, and corn growing practices that prevail annually in the highlands of Guatemala. Often the loss is from 5 to 50 per cent. Seedling injury was not common. Serious injury occurred only after permanent or crown roots had developed.

Since the corns of the Guatemalan highlands probably have been hosts to species of Diabrotica for 5000 years or more, it is a logical place to search for rootworm resistant strains of corn.

Diabrotica larvae did not feed on the roots of corn until the rainy season began. Feeding was largely confined to the permanent roots, not the seminal. Larvae were abundant in June and July on roots of corn planted in April in the Antigua region.

The permanent roots of corn planted in February and March were well developed before the rainy season began the last half of May, and escaped serious injury. There was no appreciable difference in number or time of development of permanent root whorls in the Guatemalan strains of the Early, Coast, Giganteum and Mountain groups.

Fig. 8. The comparative root injury of four plants of 92A 2s (187-2 x L 317) and one of the U.S. single cross (187-2 x L 317). The rootworm class injury of the three-way crosses varied from 1.9 to 3.6. The susceptible single cross had a root injury class of 4.0 and the 92A o.p., shown in Table 7, a 2.9.

Adults of the following species of Diabrotica and related genera have been taken on corn or emerged from cages placed over corn stubs: Diabrotica adelpha Harold, D. balteata LeConte, D. lepida (Say), D. nigrofasciata Jacoby, D. porracea Harold, D. rufomaculata Jacoby, D. sexmaculata Jacoby, D. sexmaculata Baly., D. tibialis Jacoby, D. tricolor Jacoby, Acalymma trivittata (Mann.), A. cornuta (Baly.), Amphelasma cavum cavum (Say), Andrector atrofasciatus (Jacoby), and three undescribed species or subspecies of Diabrotica. The species of Diabrotica most common as adults were D. adelpha, D. balteata and D. porracea.

The larvae of three different species of Diabrotica, D. balteata, D. porracea and D. adelpha, fed on the permanent roots in cage experiments causing typical rootworm injury.

Three hundred and ten strains or varieties of corn, collected largely in Guatemala, were tested for rootworm resistance from 1947 to 1952 inclusive. They varied in reaction from 1.5 to 5.0 on a scale of 1.0 to 5.0. Eighteen of these showed varying degrees of resistance. All, except one, belonged to the Giganteum group.

Second and third generation inbreds of 92A were as resistant as the parent variety. Resistance definitely existed in Guatemalan strains and varieties. It was heritable and transmittable in three-way hybrids made on a susceptible U.S. single cross (187-2 x L 317).

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RELATIVE TOXICITY OF LEGUME SEED PROTECTANTS TO TWO SPECIES OF RHIZOBIUM[1]

G. W. Peterson and W. F. Buchholtz

It is well known that nodulation of legume roots results in nitrogen accumulation in the plant and ultimately in the soil. Repeated demonstration of this fact has resulted in the well established practice of inoculating legume seeds with nodule-inducing bacteria.

In view of another well established practice, namely that of coating the seed of such crops as corn, oats, wheat, barley, and flax with a disinfectant or protectant fungicide, there is current interest in "seed treatments" for legume seed as a means of improving stands by protecting the germinating seed and young seedlings from soil-borne fungous pathogens. It is possible that a fungicide applied to the seed might well be bactericidal to a degree sufficient to kill or inhibit infection by nodule bacteria, especially those applied to the seed.

Several investigations have attempted to determine the effect of seed protectants on nodulation of legumes. Very few have attempted to determine the direct effect of seed protectants on the growth of legume nodule bacteria. The objective of these experiments was to determine the toxicity of some of the seed protectants commonly used in Iowa to Rhizobium meliloti and R. japonicum. By two methods the toxicities of Arasan, Arasan SF, Spergon, Spergon (wettable), and Ceresan M have been compared. Briefly, Ceresan M was found to be very toxic, Spergon and Spergon (wettable) very little if at all, with Arasan and Arasan SF intermediate.

LITERATURE REVIEW

Reports of experiments in which treated, uninoculated seed has been planted in the field or in nonsteamed soil in the greenhouse indicate that in some instances nodules are formed regardless of treatment applied to the seed, presumably by nodule-inducing bacteria already present in the soil (2, 4, 9, 12).

Tests involving the inoculation of treated seed suggest, on the one hand, that there is tolerance by the nodule bacteria to the fungicides used (3, 6, 14). On the other hand, particularly when such treated, inoculated seed has been planted in steamed soil, some seed treatment chemicals have inhibited nodulation and presumably have an adverse effect on the nodule bacteria (1, 6, 11).

Two attempts to determine the direct effect of seed protectants on Rhizobium leguminosarum have been recorded. Burton (3) grew R. leguminosarum in a suitable medium to which concentrations of Spergon from

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0.1 to 0.6 per cent had been added. Growth was markedly inhibited; at 0.6 per cent concentration there was 90 per cent inhibition. In another test Burton grew R. leguminosarum in a liquid medium until the maximum number of bacterial cells had developed. Concentrations of Spergon varying from 0.2 to 2.0 per cent were then added and the mixtures aerated for two days. At 2 per cent concentration the killing effect was about 98 per cent.

Milthorpe (11) placed different concentrations of Ceresan, Cuprox, Spergon, and Chloranil (locally prepared tetrachloro para benzoquinone) in plates inoculated with R. leguminosarum. After 5 days incubation at 28°C. the well developed colonies were counted. Ceresan was far more toxic than Cuprox, which in turn was more toxic than Spergon or Chloranil. There was the same relative order of toxicity of the fungicides in a second experiment in which the bacteria were mixed with dilutions of the above fungicides in sterile water, then plated and incubated. The LD-50 values for Ceresan and Cuprox were less than 1 milligram per milliliter and could not be determined; the LD-50 value for Chloranil was 5 and for Spergon 15 milligrams per milliliter. In a third experiment Milthorpe employed a modified cup agar test. Agar blocks containing 0.5, 1.0, 2.0 and 4.0 per cent of the respective fungicides were placed in petri dishes and covered with 10 milliliters of bacterial suspension in a nutrient agar medium. After incubation for 5 days the zones of inhibition were measured. The inhibition zones were much greater with Ceresan than with the other chemicals. The zones for Cuprox were greater than those for Chloranil, which in turn were slightly greater than those for Spergon.

The methods used by Milthorpe and the results he obtained are comparable to those reported in this paper.

MATERIALS AND METHODS

Two methods were used to expose nodule bacteria to seed protectant fungicides: the first consisted in placing fungicide-coated seeds and glass beads on agar plates seeded with bacteria and measuring the zones of inhibition of colony development around the seeds and beads; the second involved the direct addition of fungicides to a liquid culture medium inoculated with bacteria and observing bacterial growth as indicated by cloudiness of the culture.

Materials used in the tests were as follows: Alfalfa seed, Grimm variety; soybean seed, Hawkeye variety; and solid glass distillation beads, 5.6 mm. in diameter. Cultures of Rhizobium meliloti Dangeard and Rhizobium japonicum (Kirchner) Buchanan, supplied by J.C. Burton, director of research, the Nitragin Co., Inc., Milwaukee; a culture of Escherichia coli (migula) Castellani and Chalmers, supplied by the Bacteriology Department, Iowa State College; and a culture of Pythium debaryanum Hesse, supplied by Dr. Lois Tiffany, Botany Department, Iowa State College. Fungicides used were: Arasan (50 per cent tetramethyl thiuramdisulfide), Spergon (98 per cent tetrachloro para benzoquinone), Arasan SF (75 per cent tetramethyl thiuramdisulfide), Spergon (wettable) (48 per cent tetrachloro para benzoquinone), and Ceresan M (7.7 per cent ethyl mercury para toluene sulfonanilide).

Seed treatment dosages with Arasan and Spergon were: 1/4 standard,

1/2 standard, standard, and twice standard. Arasan SF, Spergon (wettable) and Ceresan M were applied at standard dosages only.

Respective standard dosages were: For alfalfa: Arasan, 4.2 oz., Spergon, 4.2 oz., and Ceresan M, 2.0 oz. per 60 lbs. of seed; Arasan SF, 3 lbs. of protectant per gallon of water applied at the rate of 1 lb. of protectant to 300 lbs. of seed; and Spergon (wettable), 2 lbs. of protectant per gallon of water, applied at the rate of 1 lb. of protectant to 415 lbs. of seed. For soybeans: Arasan, 2 oz., Spergon, 2 oz. and Ceresan M, 3/4 oz. per 60 lbs. of seed; Arasan SF, 1.5 lbs. of protectant per gallon of water, applied at the rate of 1 lb. of protectant to 1248 lbs. of seed; and Spergon (wettable), 2 lbs. of protectant per gallon of water, applied at the rate of 1 lb. of protectant per 949 lbs. of seed.

The "standard" dosages for glass beads were comparable to those for soybean seed.

Alfalfa and soybean seed were treated with the protectant dusts, Arasan, Spergon and Ceresan M, in the following manner: 50-gram samples of seed and the appropriate amount of fungicide were placed in small-mouthed bottles of 120-milliliter capacity. Seed and fungicide were uniformly rotated for 10 minutes. Stoppers were then removed from all bottles except those containing Ceresan M treated seed, which were left stoppered for 24 hours.

Alfalfa and soybean seeds were treated with slurry method protectants, Arasan SF and Spergon (wettable), in the following manner: Glass bottles identical to those used above were rinsed with distilled water; then while still wet, appropriate amounts of fungicide and water were placed in the bottles. The bottles were immediately stoppered and rotated to insure a good mixture of water and fungicide. After mixing, 50-gram samples of seed were added, and the bottles uniformly rotated for 15 minutes. At the end of this period stoppers were removed from all bottles.

Fungicides were applied to the beads in the same manner as to the seeds; however, before being coated with fungicide, the beads were boiled in 30 per cent sodium hydroxide for 2 hours to toughen the surface so the fungicide would adhere.

Yeast extract-mannitol agar, pH 7.0, was used as bacterial culture medium in all of the plate experiments. It was similar to Fred and Waksman's (5) medium No. 79, differing only in that it contained 0.1 gram of calcium carbonate instead of 3.0 grams. The plates were poured from test tubes which contained 15 milliliters of agar, to which 0.1 milliliter of a water suspension of bacteria had been added. Approximately 2 minutes after the plates were poured, treated seeds or beads were placed on the agar surface with sterile forceps. Treated alfalfa seeds were added to plates seeded with R. meliloti; treated soybean seeds were added to plates seeded with R. japonicum; and treated glass beads were added to both R. meliloti seeded plates and R. japonicum seeded plates. All plates were incubated for 72 hours at 30°C. after which zones of inhibition of bacterial colony development were measured. Measurements were of the distance between the edge of the seed and the periphery of the zone of inhibition. This method of determining toxicity by measurement of zones of inhibition in agar plates is essentially comparable to the method for assay of antibiotics (12).

In the second method of exposing nodule bacteria to fungicides, the

protectants were added to a nutrient broth medium, pH 6.8, of the following composition: 3 grams of beef extract (Bacto), 5 grams of peptone (Bacto), and 1000 milliliters of water. Protectants so added were Arasan SF, Spergon (wettable), and Ceresan M. A dilution series was made by mixing aseptically 1.0 gram of fungicide with 9 milliliters of sterile water, then transferring 1 milliliter of this mixture to 9 milliliters of sterile broth, transferring 1 milliliter of the second mixture to another 9 milliliters of broth, and so on until 6 tubes of broth contained a dilution series of the fungicide. Each tube of broth was then inoculated with 0.1 milliliter of a water suspension of bacteria and incubated at 30°C. They were observed after 72 hours to determine presence or absence of bacterial growth, indicated by cloudiness of the medium.

Pathogenicity of the Rhizobium species was determined by placing surface sterilized alfalfa and soybean seed in pots containing steamed soil, and then adding 1 milliliter of a water suspension of the appropriate species. After four weeks the plants were harvested and the roots observed for nodules. The R. japonicum culture produced nodules on soybeans. The test with R. meliloti was somewhat unsatisfactory since nodules appeared on plants in pots with and without added bacteria.

RESULTS

Zones of Inhibition of Rhizobium Around Treated Seed and Fungicide-Coated Glass Beads on Agar Plates

Alfalfa and soybean seeds treated with one-fourth standard, one-half standard, standard and twice standard dosages of Arasan and Spergon and the standard dosages of Arasan SF, Spergon (wettable) and Ceresan M, were placed on yeast extract-mannitol agar plates seeded with Rhizobium. For each treatment dosage, two treated alfalfa seeds were placed in each of three plates seeded with R. meliloti and two treated soybean seeds were placed in each of three plates seeded with R. japonicum. Three check plates contained two nontreated alfalfa seeds and three contained nontreated soybean seeds.

Glass beads coated with the same dosages of the same fungicides were likewise placed on yeast extract-mannitol agar plates seeded with Rhizobium. There was a glass bead experiment with plates seeded with R. meliloti and another with plates seeded with R. japonicum. All experiments were repeated in entirety.

In all cases, measurements of zones of inhibition of bacterial growth were made after the plates had been incubated for 72 hours at 30°C. Means of the radii of these zones are recorded in Table 1.

It is evident from the data that: 1) Larger inhibition zones were formed around seeds and beads coated with a standard dosage of Ceresan M than around those coated with the other fungicides (Fig. 1). 2) There were larger zones around seeds and beads coated with Arasan and Arasan SF than around those coated with Spergon and Spergon (wettable) (Fig. 1). 3) In general, there were larger zones around seeds and beads coated with higher than with lower dosages of Arasan; a similar trend was apparent also for Spergon. 4) Rhizobium japonicum appeared to be somewhat more sensitive to the fungicides than R. meliloti.

Although not recorded in the table, there were no zones of inhibition around nontreated seeds and noncoated beads in the check plates.

TABLE 1

Zones of inhibition of bacterial growth around seeds and glass beads coated with seed treatment fungicides and placed on yeast extract-mannitol agar seeded with Rhizobium

Fungicide	Seed	Rhizobium species	Inhibition zone radii at fungicide dosage: One-fourth standard	One-half standard	Standard	Twice standard
Arasan	Alfalfa	R. meliloti	1.2*	1.6	2.1	3.9
	Soybeans	R. japonicum	3.7	11.2	16.2	17.3
	Glass beads	R. meliloti	1.1	1.1	9.7	9.9
	" "	R. japonicum	3.1	7.4	24.5	26.1
Spergon	Alfalfa	R. meliloti	0.1	0.3	0.7	0.9
	Soybeans	R. japonicum	1.1	1.8	2.3	2.5
	Glass beads	R. meliloti	0.3	0.5	0.9	1.6
	" "	R. japonicum	0.5	0.6	0.7	2.7
Arasan SF	Alfalfa	R. meliloti			2.1	
	Soybeans	R. japonicum			7.2	
	Glass beads	R. meliloti			8.1	
	" "	R. japonicum			19.5	
Spergon (wettable)	Alfalfa	R. meliloti			0.5	
	Soybeans	R. japonicum			0.1	
	Glass beads	R. meliloti			1.1	
	" "	R. japonicum			1.8	
Ceresan M	Alfalfa	R. meliloti			7.2	
	Soybeans	R. japonicum			22.0	
	Glass beads	R. meliloti			16.1	
	" "	R. japonicum			26.3	

* Each datum is a mean of the radii (mm.) of 12 zones.

Growth of Rhizobium in Nutrient Broth with Fungicides Added

Tubes of nutrient broth containing a range of concentrations of Arasan SF, Spergon (wettable) and Ceresan M were inoculated with Rhizobium and Escherichia coli, the latter a common saprophytic species. Fungicide concentrations constituted a dilution series ranging from 10^{-1} to 10^{-6} grams of Arasan SF and Spergon (wettable) and 10^{-1} to 10^{-8} grams of Ceresan M, per 10 milliliters of nutrient broth. There were two experiments, with 3 replicate tubes per concentration per experiment, for each, R. meliloti, R. japonicum and E. coli. Growth as indicated by turbidity was observed after 72 hours incubation at 30° C. and recorded as positive if present in at least one of the three tubes at a particular fungicide dilution.

The results, presented in Table 2, were: 1) Ceresan M prevented growth of all three species of bacteria at all except the most dilute concentrations. Spergon (wettable) was inhibitive only at high concentrations,

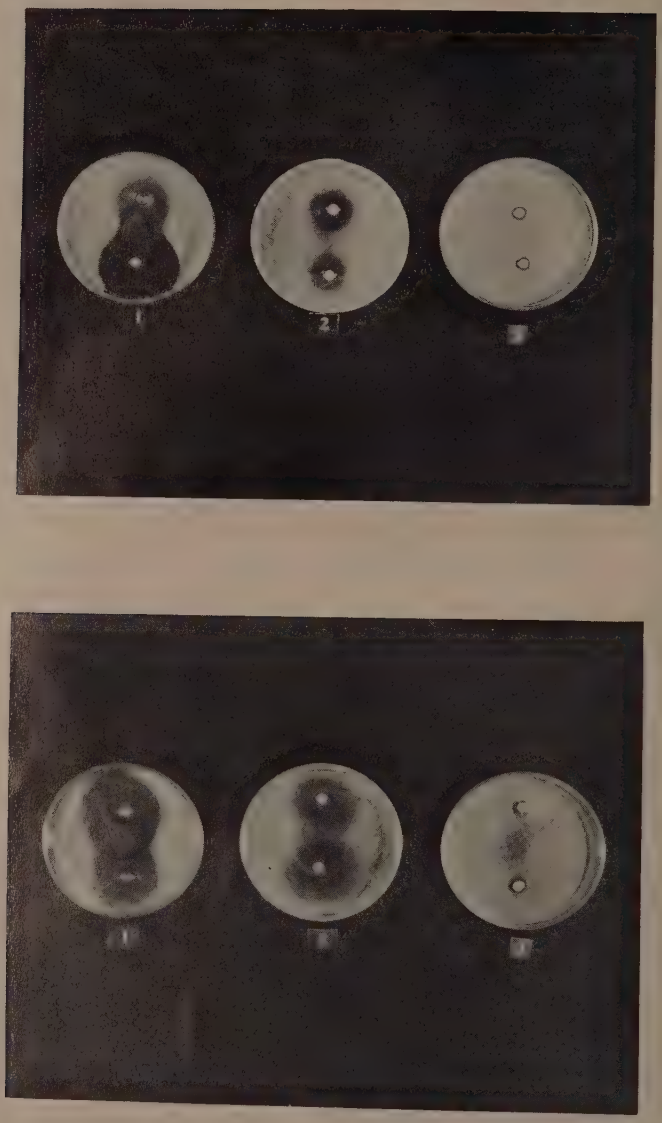

Fig. 1. Zones of inhibition of bacterial growth in yeast extract-mannitol agar plates containing glass beads coated with "standard" dosages of (1) Ceresan M, (2) Arasan and (3) Spergon, Above: Plates seeded with Rhizobium meliloti. Below: Plates seeded with R. japonicum.

while Arasan SF was intermediate. 2) In general, Rhizobium japonicum was slightly more sensitive to these fungicides than R. meliloti. Both were more sensitive than Escherichia coli. 3) The relative bactericidal effects of the three fungicides in the nutrient broth experiments and the relative sensitivities of the two species of legume nodule bacteria were comparable to those in the agar plate experiments.

TABLE 2

Growth in 72 hours by Rhizobium meliloti, R. japonicum and Escherichia coli in nutrient broth containing a range of concentrations of Arasan SF, Spergon (wettable) and

Fungicide	Bacterial species	Grams of fungicide in 10 ml. nutrient broth: 10^{-1}		10^{-2}		10^{-3}		10^{-4}		10^{-5}		10^{-6}		10^{-7}		10^{-8}		none	
		Growth in duplicate experiment: 1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Arasan SF	R. meliloti	-	-	-	-	+	+	+	+	+	+	+	+					+	+
	R. japonicum	-	-	-	-	-	-	-	-	+	-	+	+					+	+
	E. coli	-	-	-	-	+	-	+	+	+	+	+	+					+	+
Spergon (wettable)	R. meliloti	-	+	-	+	+	+	+	+	+	+	+	+					+	+
	R. japonicum	-	-	-	+	+	+	+	+	+	+	+	+					+	+
	E. coli	+	+	+	+	+	+	+	+	+	+	+	+					+	+
Ceresan M	R. meliloti	-		-		-	-	-	-	-	-	-	-	+		+		+	+
	R. japonicum	-		-		-	-	-	-	-	-	-	-	-		+		+	+
	E. coli	-		-		-	-	-	-	-	-	+	+	+		+		+	+

Growth Deterrent Effects of Fungicides Against Pythium debaryanum

Variation between the fungicides tested in their bactericidal effects prompted inquiry regarding their potential fungicidal effects. For instance, Spergon, an effective seed protectant, was only mildly bactericidal according to the two tests applied. Seed protection infers an effect against Pythium debaryanum, a common seed-rotting and seedling damping-off fungus. Accordingly, the growth deterrent effects of these same fungicides against P. debaryanum were briefly tested in the following manner.

Beads coated with "standard" dosages of Arasan, Spergon, Arasan SF, Spergon (wettable) and Ceresan M were placed on nonnutrient agar plates inoculated with P. debaryanum. A pea-sized portion of agar culture inoculum was placed in the center and three beads coated with a particular fungicide were placed near the edge of each plate. After incubation for 48 hours at 25°C., zones of inhibition of mycelial growth around the three beads were measured and recorded. For each of the fungicides and a noncoated bead check there were three plates in each of two duplicate experiments. The summarized results are presented in Table 3.

TABLE 3

Zones of inhibition of mycelial growth around glass beads coated with fungicides and placed on nonnutrient agar inoculated with Pythium debaryanum

Fungicide	Inhibition zone radii
	mm.
Arasan	13.8
Spergon	0.5
Arasan SF	15.9
Spergon (wettable)	0.0
Ceresan M	20.4
None (check)	0.0

It is evident that in plate culture Ceresan M was highly deterrent to growth of P. debaryanum while Spergon and Spergon (wettable) had little or no effect. Arasan and Arasan SF were intermediate. Although perhaps not in exact proportion, the fungicides ranked in the same order of toxicity against P. debaryanum as against the two species of Rhizobium and Escherichia coli.

SUMMARY AND CONCLUSIONS

The toxicities of Arasan, Arasan SF, Spergon, Spergon (wettable) and Ceresan M to Rhizobium meliloti and R. japonicum were determined by two methods. One method involved coating alfalfa and soybean seeds and glass beads with fungicide, placing such seeds and beads on yeast extract-mannitol agar seeded with Rhizobium and measuring zones of inhibition of bacterial colony development around them. The other method consisted in inoculating with Rhizobium a series of tubes of nutrient broth to which various amounts of fungicides had been added, and recording presence or absence of bacterial growth as evidenced by cloudiness of the medium.

Results according to both methods were that in general, Ceresan M was highly toxic to both species of Rhizobium, Spergon very little, with Arasan intermediate. Higher dosages of Arasan on seeds and beads were more toxic than lower dosages; the same trend was apparent for Spergon, at its very low toxicity level.

R. meliloti was somewhat less sensitive to all fungicides than R. japonicum; both were more sensitive than Escherichia coli.

In a test with fungicide-coated glass beads on nonnutrient agar, the fungicides were in the same order and degree of toxicity to Pythium debaryanum as to the bacteria.

It might be concluded that Ceresan M could be expected to be toxic to Rhizobium meliloti and R. japonicum applied to seed, probably sufficiently so to adversely affect nodulation; likewise Arasan and Arasan SF at higher dosages. Spergon and Spergon (wettable) can be tolerated by R. meliloti and R. japonicum, even at high dosages; therefore nodulation would probably not be seriously impeded by these fungicides. Nodulation might tend to be affected by seed treatment fungicides somewhat less adversely on alfalfa than on soybeans.

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CYTOHISTOLOGICAL RESPONSES OF PLANT MERISTEMS TO MALEIC HYDRAZIDE[1]

John B. Carlson[2]

Department of Botany and Plant Pathology,
Iowa State College, Ames, Iowa

The major biologic threats to our agriculture are weeds, insects and fungous diseases. The use of chemical plant protectants to combat these pests has increased greatly in recent years. Practical screening tests determine the pesticidal and growth regulant action of new formulations, but such tests usually reveal only the gross responses of the crop plant to the chemical. Basic work on the morphological and physiological responses of the plant is essential for a full understanding of the action of these chemicals.

The present study was undertaken to investigate the morphological responses of three plant species of major economic importance in Iowa to maleic hydrazide, a chemical that has recently been shown to have potential value as a growth inhibitor and plant protectant.

REVIEW OF PERTINENT LITERATURE

Schoene and Hoffman (39) first reported that maleic hydrazide (MH) causes a pronounced but temporary inhibition of plant growth. Since that time, an extensive literature has accumulated, pertaining to the action of MH and its possible agricultural and horticultural uses (43).

Along with the desired effects of treatment with MH, this compound often causes adverse effects such as stunting, cessation of apical growth, development of leaf abnormalities and necrosis. Many investigators have reported damage to crop plants, particularly to the seedling stage (7, 8, 15). Crafts, et al. (7) reported severe stunting and leaf malformation of several crop plants. McIlrath (29) reported that MH causes a cessation of apical stem growth in cotton. Naylor and Davis (31) studied the effect of MH on several species and showed that there is cessation of activity of the terminal meristem, cessation of elongation of the internodes, and abnormal expansion of leaves.

Possible mechanisms of the mode of action of MH have been described by several authors. Isenberg, et al. (24) reported that MH inhibits respiration through partial inactivation of one or more of the dehydrogenases.

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Naylor and Davis (32) also suggested that MH may interfere with the normal function of dehydrogenase. Carbohydrate accumulation has been noted in plants after treatment with MH (7, 9, 29). Exudation of sucrose in maize (30) and in barley (9) has been observed. Compton (5) found some inclusions in the leaves of pea seedlings, probably representing some type of polysaccharide accumulated as a result of the failure of assimilation or a reduced respiration rate or both.

Leopold and Klein (25) suggested that MH may be an anti-auxin. Currier, et al. (9) supported this by demonstrating an antagonistic action between 2,4-D and MH. They suggested that MH causes carbohydrate accumulation, whereas 2,4-D may stimulate carbohydrate depletion. However, Greulach and Atchison (22) noted that at the 1 ppm level of MH, treated onion bulbs produced many more roots than the checks, indicating a possible stimulating effect.

MH enters the plant readily through the roots and leaves and then is translocated rapidly to regions of meristematic activity (31). Currier, et al. (9) suggested that MH moves through the phloem, in which it affects tube differentiation and eventually causes necrosis, possibly accounting for the loss of apical dominance. Other authors have noted phloem necrosis following MH treatment (29, 41).

Crafts, et al. (7) suggested that MH probably acts on plant meristems. Greulach and Atchison (22) showed that MH inhibited mitosis and cell division in onion root tips in proportion to the concentration. Darlington and McLeish (12) reported that MH stopped mitosis in Vicia roots at high concentrations, and that at lower concentrations chromosomes were broken in the heterochromatic regions. They also stated that MH differs from other chromosome-breaking agents since it does not produce a sticky chromosome surface.

Greulach (21) showed that no concentration of MH inhibited growth of etiolated bean stems, indicating that MH inhibits cell division but not cell elongation. Compton (5) noted that after treatment with MH, Pisum root tips had differentiated tissues almost to the root tip, and that meristematic tissue was almost absent. She further reported that the nuclei in these tips were shrunken and the cells were extremely small. Struckmeyer (41) reported that leaves of Croft Easter Lilies treated with MH became thickened, the cells were larger and the spongy parenchyma cells were more loosely arranged. McIlrath (29) noted that cotton leaves became thicker and leathery, and that the greatest percentage of thickening occurred in the palisade and spongy mesophyll layers. Currier, et al. (9) found that barley leaves became thicker and more brittle following MH treatment.

MATERIAL AND METHODS

Three crop species were used in this study: Oats, Avena sativa L. var. Shelby; soybean, Glycine max (L.) Merr. var. Hawkeye; and maize, Zea mays L., yellow dent hybrid Ohio C-92.

The crystalline maleic hydrazide (MH) used in all treatments was supplied by the Naugatuck Chemical Division of the U.S. Rubber Company.

Seeds of oats and maize were treated by soaking for 48 hours at 8°C. in aqueous solutions of MH. Prolonged soaking seriously reduced the germination of soybeans. However, satisfactory results were obtained

by reducing the soaking time to 24 hours. Concentrations of 0.025, 0.05, 0.1 and 0.2 per cent MH were used, as well as check treatment in water.

After soaking, the seeds were germinated by one of two methods. Some seeds were planted in four-inch pots filled with a fertilized loam and placed in the greenhouse. The other seeds were planted in moist Sphagnum moss in plastic germination chambers which were placed into a germinator kept at 30°C. during the day and 20°C. during the night. Daily observations were made on the germinating seeds during early seedling development.

Stem tips and root tips were periodically collected and fixed in Craf III and Allen-Bouin II, respectively, and processed by the dioxan-normal butyl alcohol method (37). For histological study, slides were stained with safranin-fast green and for cytological study with iron-hematoxylin, gentian violet and Feulgen reaction. The Erliki-Zirkle formulation was used in basic fixation for the study of mitochondria (37).

EXPERIMENTAL RESULTS

Gross Morphological Responses

The initial response of seeds of soybeans, oats and maize to treatment with MH is the retardation of growth of the radicle and plumule. This retardation occurs after presoaking seeds in aqueous solutions of MH, or after planting directly onto a substrate wet with the MH solution. Increasing the concentration of MH increases the inhibitory effect (Fig. 1). At the concentrations of MH used, the inhibition of growth of presoaked oats, maize and soybeans is usually temporary, and most of the seedlings recover and subsequently develop normally. Continuous exposure of all three species to 0.025 per cent MH and higher concentrations produces permanent inhibition and eventual death.

Tests were made to determine the effect of MH upon the germination percentage of maize, oat and soybean seeds. Seeds were soaked at 8°C. in concentrations of 0.00, 0.025, 0.05, 0.1, and 0.2 per cent aqueous solutions of MH. Oats and maize were soaked for 48 hours, soybeans for 24 hours. One hundred seeds of each species were used at each concentration of MH. The soaked seeds and 100 unsoaked seeds were then rolled into paper towels and placed in a 20° - 30°C. germinator. Three replications were used for maize, and four replications each for oats and soybeans. Germination counts were made at eight days and were based on radicle emergence. The average germination percentage of all replications is presented in Table 1.

A comparison of the means of the unsoaked series with the soaked series indicates that soaking caused a decrease in germination, significant at the 1 per cent level.

Analyses of variance of the germination percentage of the soaked seeds were calculated and are presented in Table 2.

In no case was the F value significant, indicating that there was no change in germination percentage due to the MH treatments at the concentrations used.

Seven days after planting, oats treated with 0.1 and 0.2 per cent have shoots 1/5 to 1/10 as long as those of untreated plants. The coleoptile

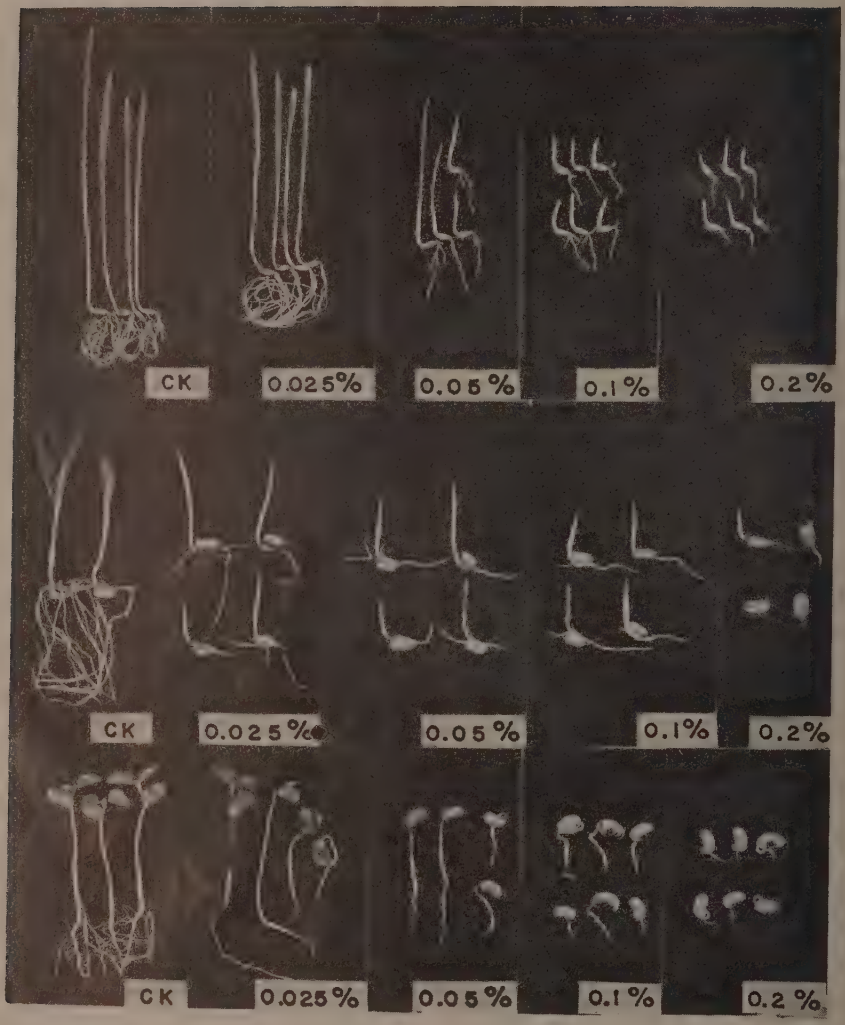


Fig. 1. Top row. Oat seedlings seven days after planting; seeds pre-soaked 48 hours in water or in MH solutions at the stated concentrations. (0.23x)

Center row. Maize seedlings seven days after planting; seeds pre-soaked 48 hours in water or in MH solutions at the stated concentrations. (0.23x)

Bottom row. Soybean seedlings ten days after planting; seeds pre-soaked 24 hours in water or in MH solutions at the stated concentrations. (0.23x)

elongates slowly, becomes somewhat thicker, and remains whitish in color. The enclosed plumule of two seminal leaves grows more slowly than the coleoptile, which thus remains relatively empty.

TABLE 1

Average germination percentage of soaked and unsoaked seeds.

	Unsoaked	Soaked				
		H_2O	Per cent concentrations of MH			
			0.025	0.05	0.1	0.2
Soybeans	96.8	61.8	63.0	67.8	69.0	65.0
Maize	98.3	86.3	87.0	83.7	82.7	81.7
Oats	90.8	77.5	76.5	70.8	75.0	76.5

TABLE 2

Analyses of variance of germination percentage of soaked seeds.

	Soybeans		Maize		Oats	
	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.
Replications	3	94.47	2	24.05	3	19.77
Treatments	4	37.68	4	13.56	4	28.50
Error	12	46.51	8	12.86	12	29.03

Root growth of treated oats is also strongly retarded at concentrations of 0.1 and 0.2 per cent MH. At seven days the radicle is 1/10 to 1/15 the length of the radicles of untreated plants. Seminal roots are reduced or lacking, and no secondary or adventitious roots develop. Many root hairs develop to the tip of the radicles and seminal roots. This is in contrast to the normal roots in which the root hairs develop a centimeter or more back from the tip. The lowest millimeter of the root tip often becomes somewhat bulbous, subtended by the sharp-pointed root cap. These root tips later become necrotic.

Maize is more tolerant than oats to MH, but both respond in a similar manner. Seven days after germination, the shoot of maize treated with 0.1 and 0.2 per cent MH is short, about 1/4 as long as in check plants. The coleoptile is somewhat thicker than that of the control. Freehand sections show that the distal portion of the coleoptile is empty. In the basal region, the five seminal leaves remain relatively undeveloped. No new leaves are produced during this interval.

The radicle and seminal roots of treated maize develop slowly, and secondary roots are lacking. The radicles are about 1/10 as long as normal ones. Root hairs are less prominent than in the check, but they extend to the extreme tip of the root. Some of the root tips are bulbous and have a sharp tip as in oats. These root tips eventually die.

Fig. 2. Longisection of oat root tip, untreated. (42x)

Fig. 3. Longisection of maize root tip, untreated. (42x)

Fig. 4. Longisection of soybean root tip, untreated. (42x)

Fig. 5. Longisection of oat root tip from seedling plant seven days after planting; seeds pre-soaked in 0.1 per cent MH. (42x)

Fig. 6. Longisection of maize root tip from seedling plant seven days after planting; seeds pre-soaked in 0.1 per cent MH. (42x)

Fig. 7. Longisection of soybean root tip from seedling plant ten days after planting; seed pre-soaked in 0.1 per cent MH. (42x)

d = dermatogen

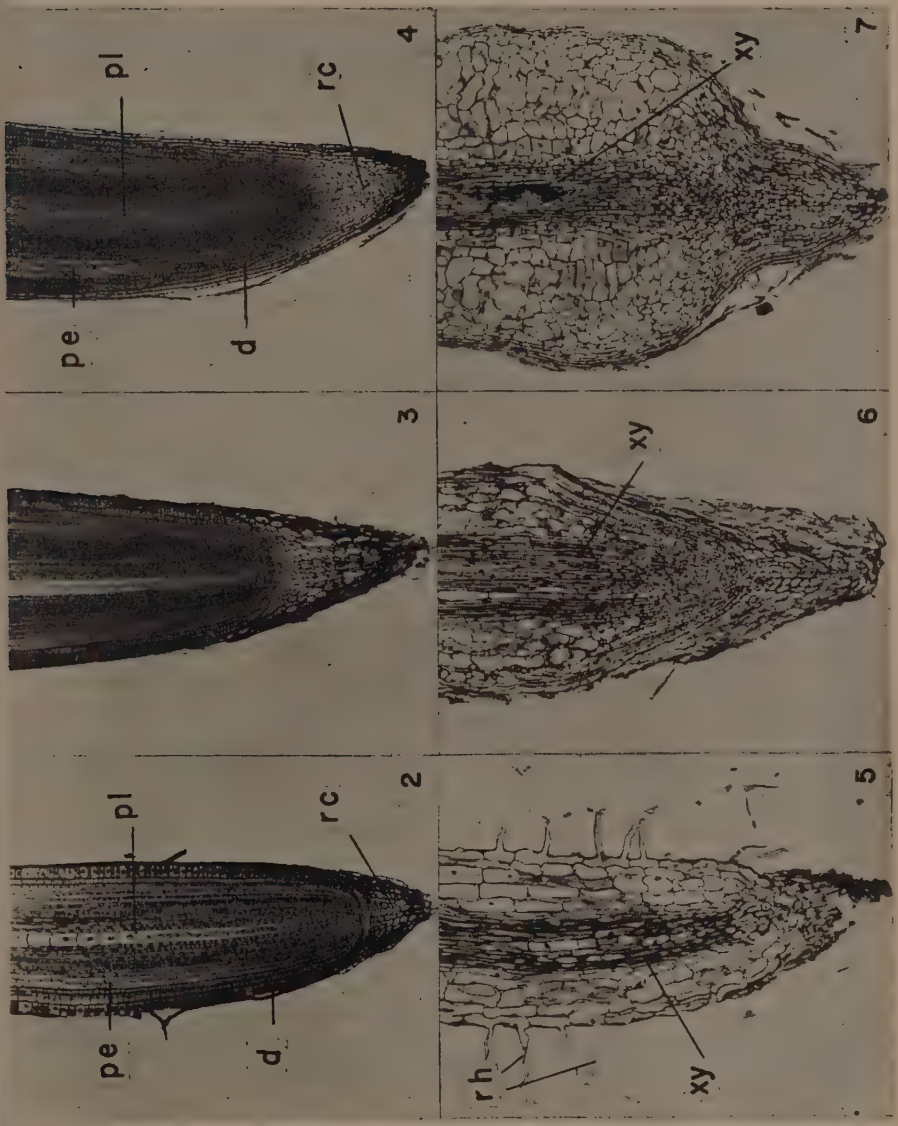
pe = periblem

pl = plerome

rc = root cap

rh = root hair

xy = mature xylem elements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Seven days after treatment with 0.1 and 0.2 per cent MH, growth of the soybean seedling is much retarded. Little or no elongation of the epicotyl or hypocotyl occurs. The plumule remains small and tightly enclosed by the cotyledons. The untreated plants are 4 - 6 cm. tall at seven days, and the simple primary leaves are expanded. The treated plants have no externally visible stem abnormalities, other than reducation of length. Root growth is very much inhibited. The radicle elongates slightly, to about 1/10 normal length, and no secondary roots are formed. The root tips are bulbous with sharp tips but no root hairs are formed. Many of these bulbous root tips are necrotic.

Histological Responses

Root development

Root tips emerging from treated seeds exhibit several histological abnormalities (Figs. 2-7). In the three species studied, the root tips are larger in diameter than normal and often have a prominent bulge in the histogen region. The increase in thickness is due mainly to extensive vacuolation and enlargement of the periblem cells. As a result of their abnormal enlargement, the periblem cells lose much surface contact with adjacent cells, the cells become loosely packed, and large intercellular spaces are formed. The cells of the protoderm and plerome also become much enlarged and vacuolate, and contribute to the thickening of the root tip (Figs. 5, 6, 7). Soybean root tips exhibit the greatest thickening, and some specimens become two or three times normal diameter. In treated soybeans, considerable quantities of starch may accumulate in the cortical cells, but this has not been consistently observed in all roots. No starch was noted in root tips of treated corn and oats.

The terminal region of the root tip is not as greatly enlarged as the region immediately above. Considerable vacuolation and cell enlargement occurs in the calyptrogen, and in the cells that initiate the dermatogen, periblem and plerome (Figs. 5, 6, 7). The sharp-pointed root cap shows relatively little change from normal, although considerable cell enlargement is evident.

Many root hairs are produced to the end of the root tip. These root hairs appear to be no longer than those on normal roots but are larger in diameter. Their position is unique, since root hairs of these species normally occur one or more centimeters back from the tip. In some cases, root hairs are present to the extreme tip (Fig. 5). Oat root tips exhibit this abnormal root hair development to a greater extent than maize or soybeans.

The vascular cylinder or stele of the treated root tip is also abnormal. The region of maturity, which in normal roots is one to several centimeters back from the tip, is projected to the end of the root tip of treated seeds. In all three species, mature metaxylem and protoxylem elements with lignified secondary thickenings have been found within 0.1 mm. of the tip. These mature vascular elements appear normal in structure.

Root tips which reach this stage of histological maturity have no residual meristematic tissue and soon die. The first visible stage of necrosis in the root tips is the disintegration of the protoplast and later of the cell walls of the cortical cells. This breakdown produces a cavity bounded

externally by the still-intact epidermal cells. The differentiated, highly lignified stele persists and projects into this cavity. The epidermal cells collapse and break down in the final stages of necrosis.

Many of the roots in the species studied, particularly those treated at lower concentrations of MH, do not exhibit these abnormalities and grow normally, although somewhat slowly.

Stem development

Since maize and oats have essentially similar shoot development, they will be considered together. The emergent shoots of maize and oats treated with 0.1 and 0.2 per cent MH are short and broad and are retarded in linear growth. The increase in thickness is due to a general enlargement and vacuolation of the cells of the coleoptile, seminal leaves, stem internodes, and growing point. These organs and tissues are considerably broader than normal. This cell enlargement is more pronounced in oats than in maize, and the coleoptiles of both species exhibit greater enlargement than the other organs (Figs. 8 - 11).

No leaf primordia are produced by the stem apices of treated oats and maize during the seven days after planting. At the end of this time, only the two seminal leaves of the mature oat embryo are present (Fig. 9). In the untreated plantings three or four leaves are present, one or two of which had been produced after germination. The first and second tiller primordia are generally present in the check plantings (Fig. 8).

In treated maize only the five seminal leaves are present at seven days, whereas the untreated maize seedlings have six or seven leaves. Two or three axillary bud primordia are present in the checks, and none are apparent in the treated plants (Figs. 10, 11).

Small amounts of starch are present in untreated oat and maize seedlings, particularly in the coleoptile, the youngest leaves and at the uppermost nodes. There is considerably more starch present in all the parenchymatous tissues of the shoots of treated maize and oats, but it is concentrated mainly in the lower region of the coleoptile and in the stem (Figs. 9, 11). Starch determinations were made with iodine and with the use of the polarizing microscope.

The response of the vascular system in the stem is similar to that of the roots. The provascular strands which were present in the embryonic stem axis and appendages are all mature at seven days in the treated plantings, but no new ones are produced. The protoxylem elements have well developed annular or spiral thickenings and the metaxylem elements have lignified, pitted secondary walls. The phloem is in varying degrees of collapse in the oat leaf bundles, but this was not noted in maize.

The growth of the stem and plumule of soybeans treated with 0.1 and 0.2 per cent MH is retarded (Fig. 12, 13). The cells of the pith, cortex and epidermis undergo considerable enlargement and vacuolation, as do the cells of the leaf primordia and the dome-shaped apical meristem. The plumular axis becomes thicker and broader as a result of this cell expansion.

Ten days after germination, the treated soybean plants have only the two simple plumular leaves and the primordium of the first trifoliate leaf, all of which were present in the embryo. No axillary buds are evident at ten days (Fig. 13). In the untreated soybeans, there are in addition four

Fig. 8. Longisection of stem tip of oat seedling seven days after planting; seeds pre-soaked in water. (50x)

Fig. 9. Longisection of stem tip of oat seedling seven days after planting; seeds pre-soaked in 0.1 per cent MH. (50x)

Fig. 10. Longisection of stem tip of maize seedling seven days after planting; seeds pre-soaked in water. (50x)

Fig. 11. Longisection of stem tip of maize seedling seven days after planting; seeds pre-soaked in 0.1 per cent MH. (50x)

Fig. 12. Longisection of stem tip of soybean seedling ten days after planting; seed pre-soaked in water. (50x)

Fig. 13. Longisection of stem tip of soybean seedling ten days after planting; seeds pre-soaked in 0.1 per cent MH. (50x)

a = stem apex

ax = axillary bud

co = coleoptile

eh = epidermal hair

lp = leaf primordium

p = provascular strand

sp = simple primary leaf

st = starch

tl = trifoliate leaf

v = mature vascular tissue


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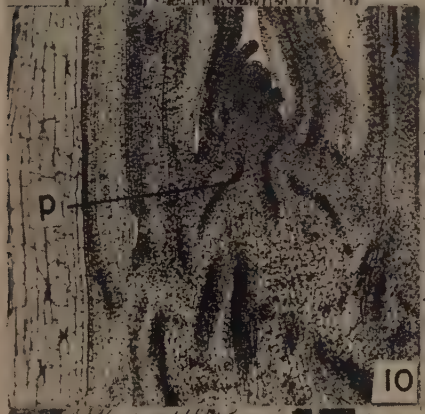
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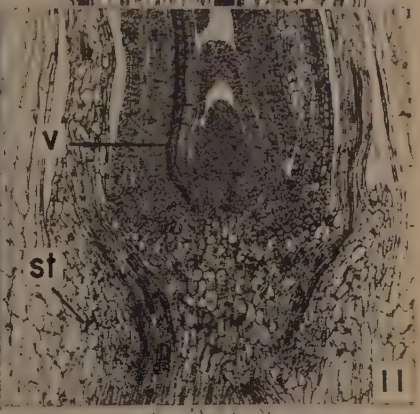
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trifoliate leaves and three or four axillary buds (Fig. 12). The treatment evidently inhibits the formation of new leaf primordia and axillary buds during the ten days after germination.

Starch is present in small quantities in the parenchyma of the older leaves and stem internodes in untreated soybeans. In treated plants, considerably more starch accumulates in all stem and leaf tissues, including the youngest leaf primordia.

Epidermal hairs, which in the normal soybean seedlings occur on the older leaves and stems, are produced on treated soybeans on all leaf and stem surfaces, including the tunica layer of the youngest leaf primordium and of the apical dome (Fig. 13). These epidermal hairs appear to be normal in structure.

The vascular system of treated soybeans matures in a manner similar to that of treated oats and maize. All provascular strands that were present in the embryo become fully differentiated by ten days, but no new provascular strands are initiated. In the section adjacent to that shown in Fig. 13, lignified, pitted metaxylem elements extend into the youngest leaf primordium to within a few cells of the distal end.

Cytological Responses

In seeds of maize, oats and soybeans treated with 0.1 per cent MH, germination is delayed one or two days. The embryos imbibe water, the cells enlarge and growth proceeds slowly. However, mitosis is not resumed for several days, or may be completely inhibited, Mitosis is first resumed in oat stem tips to a limited extent four or five days after planting, whereas no mitosis was observed in maize stem tips by seven days, or in soybeans by ten days.

For several days after germination, growth of these seedlings is evidently due entirely to enlargement of the cells which were present in the dormant embryo. The cells enlarge until they become about twice normal size. This enlargement often results in the separation of cells and an increase in intercellular space, particularly in the corpus of the apex and in the cortex of older regions (Figs. 5, 6, 7, 9, 11, 13).

The cytoplasm becomes very vacuolate in these distended cells, in contrast to the dense cytoplasm in meristematic cells of untreated seedlings. The vacuoles may be relatively few in number and very large, or they may be numerous and proportionately small. The structure of the cytoplasm, such as granularity and stainability, is not visibly altered. The nuclei enlarge as much as twice normal diameter, they stain less readily and are less dense in appearance than normal nuclei.

When mitosis is resumed in oat stem tips, abnormalities of the process become evident. The frequency of mitotic figures in treated oats is very low. A single section such as that shown in Fig. 9 may have only one to five or six mitotic figures, or there may be none, whereas a section of an untreated oat stem tip collected at the same time may have from 20 - 40 figures.

Prophase figures do not exhibit abnormalities of structure or behavior. Abnormal chromosome behavior becomes evident in metaphase and in later stages (Figs. 14-33). In metaphase, irregular orientation and fragmentation of the chromosomes occurs, with entire chromosomes and

Figs. 14-26. Resumption of mitosis in the stem tips of oat seedlings four and five days after planting; seeds pre-soaked in 0.1 per cent MH. (900x)

Figs. 14-16. Metaphase figures with fragments and chromosomes on the spindle and free in the cytoplasm.

Fig. 17. Late anaphase with bridges, fragments and a large mass of chromosomes free from the upper polar group.

Fig. 18. Anaphase with extreme fragmentation.

Fig. 19. Anaphase with a large mass of chromosomes left at the plate.

Fig. 20. Anaphase with bridges. Micronuclei forming in the cytoplasm.

Fig. 21. Late anaphase with many micronuclei.

Fig. 22. Late telophase with bridges persisting after formation of the cell plate.

Figs. 23-24. End of cytokinesis.

Figs. 25-26. Multinucleate cells.

Figs. 27 - 33. Resumption of mitosis in the stem tips of oat seedlings four and five days after planting; seeds pre-soaked in 0.1 per cent MH. (500x)

Fig. 27. Metaphase with fragments in the cytoplasm. Note two multinucleate cells.

Fig. 28. Metaphase with fragments and chromosomes free in the cytoplasm.

Fig. 29. Same as Fig. 28 at a different focus. Note the long chromosome extending from the plate.

Fig. 30. Anaphase with a large mass of chromosomes left at the plate.

Fig. 31. Anaphase with fragments in the cytoplasm.

Fig. 32. Anaphase with a wide bridge and fragments.

Fig. 33. Late anaphase with a fragment on the spindle. Note the cell plate.

Figs. 34 - 40. Mitosis in root tips four hours after transfer of germinating seeds to MH-soaked blotters. (500x)

Fig. 34. Maize. Metaphase with two persisting nucleoli extending from the plate.

Fig. 35. Maize. Two metaphase figures, one with a free chromosome, the other with a large persisting nucleolus.

Fig. 36. Maize. Two anaphase figures with bridges.

Figs. 37-39. Soybean. Metaphase figures with persisting nucleoli. Note position of some nucleoli at the poles.

Fig. 40. Soybean. Late anaphase with one nucleolus at each pole.

Fig. 41. Mitochondria in maize root tip, untreated. (500x)

Fig. 42. Same, after 72 hours continuous exposure to MH. Note the large vacuoles. (500x)


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fragments of chromosomes scattered along the spindle or free in the cytoplasm (Figs. 14-16, 27-29). These small fragments and chromosomes give the Feulgen reaction for chromatin. Most of them appear to be single, but some are double. They often tend to accumulate in the cytoplasm in the ends of the cell (Fig. 20). Very long chromosomes may extend from the metaphase plate, along the spindle and out into the cytoplasm (Figs. 15, 28, 29).

In anaphase, the fragments, or entire chromosomes, may lag on the spindle or wander into the cytoplasm (Figs. 18-20, 30-33). Bridges occur frequently, and may be rather narrow or very broad (Figs. 20, 21, 32). In some cases, large masses of chromosomes may be left at the metaphase plate (Figs. 19, 30). Excessive fragmentation may occur, fragments and chromosomes become scattered along the spindle, and no polar groups form (Fig. 18). Some micronuclei become apparent in late anaphase (Fig. 20). Large masses of chromosomes may break away from the polar groups and become free of the spindle (Fig. 17).

Many micronuclei occur in telophase, incorporating fragments, entire chromosomes, and masses of chromosomes. A cell may have several micronuclei and a well defined telophase figure (Fig. 21). Bridges are also apparent, and often appear intact even after the cell plate has formed (Figs. 17, 22). Nuclear membranes form around the polar groups in late telophase (Fig. 22). The cell plate develops in a normal manner from the center of the phragmoplast and extends outward. The resultant multinucleate daughter cells have nuclei of various sizes (Figs. 23-27).

The foregoing observations describe the responses of seedlings developing from seeds treated while dormant. For comparison, an experiment was designed to investigate the effects of MH on tissues that were treated during active growth. Seeds of maize, oats and soybeans were germinated on wet blotting paper at room temperature. When the primary roots were about two centimeters in length, the seeds were transferred to germination chambers kept at 25°C. and containing blotters wet with 0.05 per cent MH. Collections of root tips were made at 0, 4, 8, 12, 24, 48, and 72 hours.

The incidence of mitotic figures was determined by making counts of all cells in a 430x field, on three adjacent longisections at the distal end of each of four root tips. The data are in Table 3 and are graphically shown in Fig. 43.

At the time of transfer to MH, the root tips showed an abundance of mitotic figures. The frequency of mitosis decreased sharply the first four hours and continued to decrease until all mitotic activity stopped by 48 or 72 hours.

After exposire to MH for four or more hours, mitotic irregularities were noted in the three species. Chromosome hreakage occurred in maize and oats but not in soybeans (Fig. 35). The behavior of the chromosomes and fragments is similar to that described for oat stem tips, but breakage was not as frequent. Chromosome bridges were found only in maize (Fig. 36).

After four hours exposure of root tips to MH, mitotic figures in maize and soybeans showed that the nucleoli are often retained through the entire mitotic cycle (Figs. 34, 35, 37-40). Four to eight hours after treatment, about 20-30 per cent of the mitoses in maize, and 45-60 per cent of the

TABLE 3

Number of mitotic figures per 100 root tip cells after continuous exposure to 0.05 per cent MH.

Hours of exposure	Mitoses per 100 cells		
	Oats	Maize	Soybeans
0	8.6 ± 0.76*	5.0 ± 0.47	8.0 ± 1.21
4	2.9 ± 0.75	2.5 ± 0.43	2.9 ± 0.57
8	0.5 ± 0.15	2.2 ± 0.03	1.3 ± 0.38
12	0.8 ± 0.27	1.1 ± 0.31	0.8 ± 0.11
24	0.2 ± 0.05	0.1 ± 0.04	0.3 ± 0.14
48	0.0 ± 0.00	0.2 ± 0.13	0.0 ± 0.00
72	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00

* ± one standard error with 3 d.f.

mitoses in soybeans have persisting nucleoli. There may be one or two nucleoli present, attached by a chromosome to the metaphase plate, unattached and free in the cytoplasm, or at the poles. The nucleolus may appear dumbbell-shaped and lie perpendicularly across the plate and directed toward the poles. These nucleoli may then break loose and migrate to the poles. The nucleolar mass may also be seen stretched between the anaphase groups, forming a "nucleolar" bridge. More often, however, the nucleoli are found at the poles in anaphase and telophase. These nucleoli, which do not give a positive Feulgen reaction, are excluded from the daughter nuclei, become smaller, and dissolve into the cytoplasm. No persisting nucleoli were seen in oat root tips.

Measurements were made of the volume of the nucleolus in interphase cells of untreated and treated root tips to determine whether a measurable change of volume accounts for the persistence of the nucleoli. The data are given in Table 4, and are represented graphically in Figs. 44 and 45.

The nucleolar volumes in all root tip tissues except the root cap increase sharply during the first four hours exposure to MH. The volumes of the plerome nucleoli continue to increase to the end of 72 hours. In the other tissues, a peak is reached followed by a decline. In general, the increase in nucleolar volume is greater in soybeans than in maize.

The root tips fixed in the Erliki-Zirkle formulation were studied to determine if any visible changes took place in the mitochondriome following exposure to MH, which could give a possible clue to the mode of respiration inhibition. No changes were noted other than spatial displacement of the mitochondria caused by the vacuolation of the cells of treated root tips (Figs. 41, 42).

Starch accumulates in maize and soybean root tips after exposure to MH. In untreated maize root tips considerable starch is present in the central part of the root cap and somewhat less starch is present in the cortex and pith. A large accumulation of starch occurs shortly after treatment, builds up in the root cap, cortex and pith, and reaches a peak at eight hours. The starch content then diminishes until none is present at 72 hours. Soybean root tips respond in a similar manner. Some starch

TABLE 4

Average nucleolar volumes in four root tip tissues after continuous exposure to 0.05 per cent MH.

Hours of exposure	Average nucleolar volume in cubic microns			
	Protoderm	Periblem	Plerome	Root cap
		MAIZE		
0	33.9 ± 3.06*	33.4 ± 3.40	23.9 ± 2.39	12.2 ± 1.22
4	52.0 ± 2.63	43.1 ± 3.76	35.1 ± 3.55	13.4 ± 1.20
8	36.7 ± 3.50	40.7 ± 6.48	28.2 ± 2.02	13.3 ± 1.07
24	25.1 ± 2.83	37.0 ± 2.70	34.1 ± 3.39	16.1 ± 3.35
72	14.8 ± 2.07	26.0 ± 6.17	38.3 ± 1.71	14.0 ± 2.07
		SOYBEANS		
0	30.3 ± 1.97	37.5 ± 3.57	25.9 ± 2.38	2.2 ± 0.39
4	35.6 ± 4.41	48.6 ± 4.71	34.9 ± 4.68	2.1 ± 0.19
8	37.7 ± 4.32	50.5 ± 5.23	38.4 ± 5.58	2.6 ± 0.47
24	56.5 ± 6.57	77.3 ± 3.80	41.2 ± 4.65	2.0 ± 0.37
72	33.8 ± 1.77	50.9 ± 5.23	42.3 ± 2.22	1.4 ± 0.38

* ± one standard error with 3 d.f.

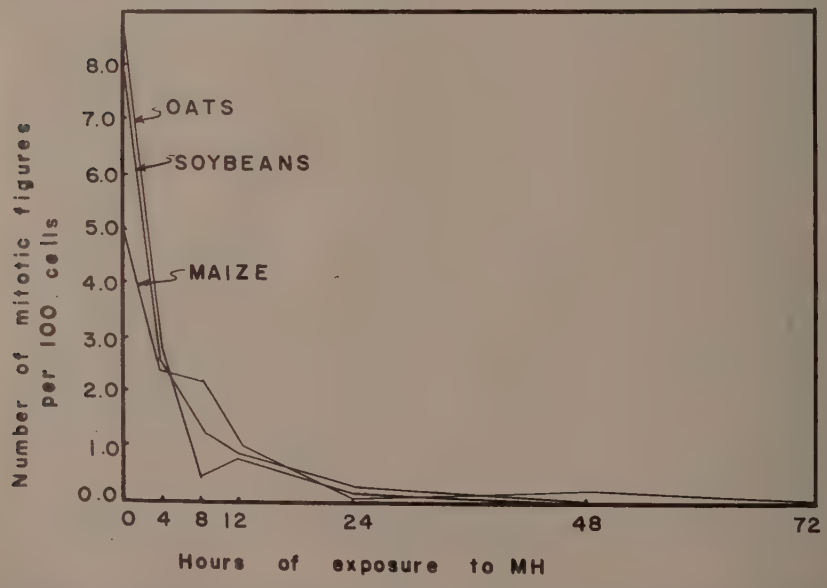

Fig. 43. Effect of continuous exposure to MH upon frequency of mitosis in root tips.

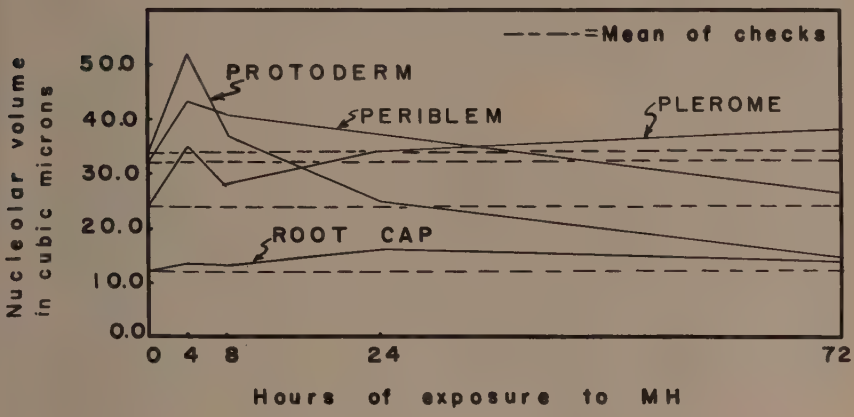

Fig. 44. Effect of continuous exposure to MH upon nucleolar volume in maize root tips.

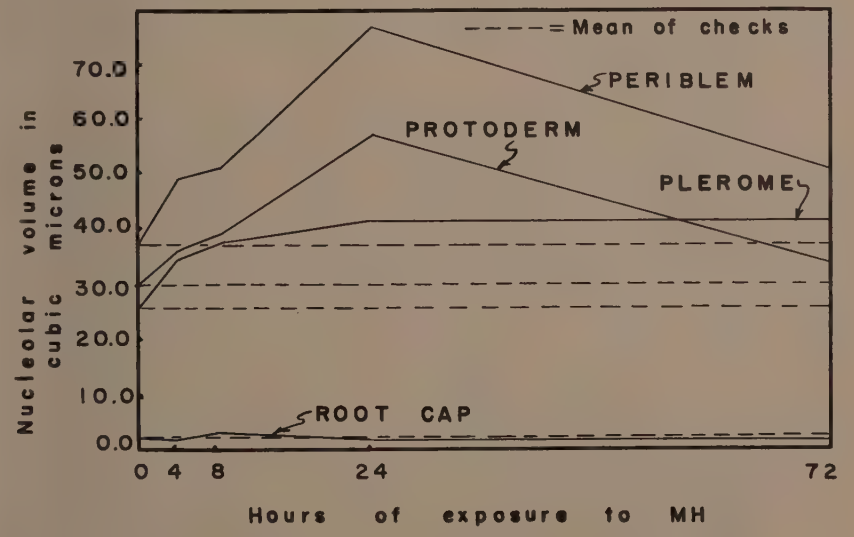

Fig. 45. Effect of continuous exposure to MH upon nucleolar volume in soybean root tips.

is present in the root cap and cortex of untreated soybean root tips. Much more starch accumulates after treatment, reaches a peak at eight hours and then diminishes until only a trace is present at 72 hours. No starch was noted in the treated oat root tips.

Extensive cell enlargement, the formation of root hairs to the extreme tip, and maturation of vascular tissues in meristems become evident, similar to the responses of seedlings developing from seeds treated while dormant.

DISCUSSION

The present report is in substantial agreement with papers that have described some features of the responses of plants to maleic hydrazide (7, 22, 31, 39). Several authors have reported the abnormal thickening of organs of treated plants by excessive enlargement and vacuolation of cells (9, 29, 31, 41). However, Compton (5) reported that treated Pisum root tips had extremely small cells and shrunken nuclei. The present study has shown that all categories of seedling organs, coleoptile, leaves, stems and roots, exhibit an enlargement and vacuolation response to MH. Other chemical agents are known to produce similar effects (14, 26, 28, 34, 35, 36).

The present study supports and amplifies Compton's note that tissue differentiation occurs almost to the root tip after treatment with MH. Vascular differentiation in root tips has been reported by other workers using agents active upon meristematic tissues (14, 28, 40). Tissue differentiation in meristems following MH treatment is also evidenced by the formation of root hairs to the end of the root, and by epidermal hairs on the youngest leaf primordium and on the apical meristem of soybean. As a result of this differentiation the root tips and stem tips have no remaining meristematic tissue, thus no more tissues or organs are produced, and the tips remain static and eventually die.

The accumulation of starch in various tissues after MH treatment is in agreement with several authors who report the increase of sucrose (9, 29, 30), starch (29), fructosan (9), carbohydrate (7), and polysaccharide (5) in treated plants. This accumulation is probably the result of a reduced respiration rate, or to phloem necrosis which interferes with translocation (5, 29).

The inhibition of mitosis by MH reported in the literature also occurs in the species described herein (5, 12, 21, 22). Cell enlargement occurs regardless of the concentration of MH, as previously reported (21). The phenomenon is similar to the inhibition of mitosis by other chemicals (3, 14, 16, 34).

The chromosome bridges observed in the present material suggest chromosome stickiness. This contrasts with the report by Darlington and McLeish (12) that in Vicia root tips, MH does not produce a sticky chromosome surface.

Breakage of chromosomes in Vicia after MH treatment has been reported (12). Two of the species used in the present study, oats and maize also exhibit chromosome breakage. Maleic hydrazide may be classified with several agents that have a similar ability to break chromosomes. Among these are P^{32} (1), C^{14} (4), oxygen (6), phenols (27), ultrasonic vibrations (33), chloranil (44), formalin (2), and many others (11).

It has been suggested that chromosomes may be broken during interphase by X-rays or mustard gas when these agents cause a breakage of the chromosome thread through some protein fiber effect (11). The chromosome breakage by MH might be due to a similar interphase breakage of the chromosome thread, or the breakage may be due to the stickiness of the chromosomes. The stickiness might be caused by dissolution of the chromosome pellicle, or to an excess nucleic acid charge on the chromosomes (11).

The persistence of nucleoli as a result of MH treatment has not been reported previously. However, persistence of nucleoli is of common natural occurrence in a wide range of plants, including algae, club mosses, ferns, and many flowering plants (10, 18, 19, 20, 23, 38). The behavior of the nucleoli is similar to that described by Frew and Bowen (19) in Cucurbita, and by Tjio (42) in Ceiba pentandra. Fischer (17) showed that the size of the nucleolus is influenced by nutrition. He found that as the food supply is reduced, the nucleolar volume decreases, and as food supply increases the nucleoli enlarge. The increase in nucleolar volume demonstrated in the present study may be correlated with the accumulation of starch in the root tips.

Ehrenberg (13) stated that the nucleolus is in equilibrium with the nucleus, but when the nuclear membrane breaks down, the nucleolus dissolves into the cytoplasm on a concentration gradient. When the cytoplasm reaches saturation, the nucleolar material remaining, if any, constitutes the persisting nucleoli. This may explain the persistence of nucleoli in MH-treated material, since the nucleoli in treated root tips are larger than normal, and could saturate the cytoplasm before being completely dissolved.

Erickson and Rosen (16) showed that protoanemonin causes the disappearance of mitochondria in Zea. MH does not produce this effect.

SUMMARY

A study was made of the morphological and cytological responses of seedlings of three crop species, oats, maize and soybeans, to maleic hydrazide (MH).

Seeds of these plants were soaked in aqueous solutions of 0.025, 0.05, 0.1 and 0.2 per cent MH and germinated. Untreated seeds were also germinated and then treated with MH.

No decrease in germination resulted from MH treatment, but there is a one- or two-day delay in germination.

Both root and shoot growth are retarded, root growth more than shoot growth. The growth of oats is inhibited most, soybeans next and maize least with the concentrations of MH used.

An increase in size occurs in root and plumular organs. This enlargement is due to extensive vacuolation and enlargement of the cells of all tissues of the seedling, associated with an increase in intercellular space. The nuclei of the enlarged cells are also abnormally large.

Starch accumulates in the tissues of the root, stem, and leaf.

The meristematic tissues take on an abnormally mature appearance. Roots may develop hairs to the tip and have mature vascular tissues up to 0.1 mm. of the tip. Mature vascular tissues occur in leaf primordia.

Soybean stem tips have epidermal hairs on the tunica of the youngest leaf and on the apical meristem. The apical meristems of the stem and root lose their meristematic function and produce no new tissues or organs.

Mitosis is inhibited by MH treatment. Frequency of mitosis in actively growing tissues drops quickly following exposure to MH.

When mitosis is resumed, mitotic abnormalities become apparent. Extensive breakage of chromosomes occurs. Chromosomes and fragments of chromosomes are lost on the spindle or move into the cytoplasm where they form micronuclei. Many bridges are formed, which may indicate a stickiness of the chromosomes. It is suggested that the chromosome breakage may be due to this stickiness. Cytokinesis appears to be normal. However, the daughter cells are multinucleate.

The nucleolar volume increases sharply after MH treatment. This is attributed to an increase in the food supply of the root tip, as shown by starch accumulation. Persisting nucleoli are found throughout the entire mitotic cycle, presumably a direct result of the increased nucleolar volume.

There is no visible change in the mitochondria after MH treatment.

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ISOLATION OF CELLULOSE-DIGESTING MICROORGANISMS OF THE RUMEN[1]

Warren D. Kitts[2], P. H. Carr, and L. A. Underkofler

Department of Chemistry, Iowa State College,
Ames, Iowa

The anaerobic cellulolytic bacteria which have been isolated fall into five categories, actinomycetes, thermophilic spore-formers, nonspore-forming rods and cocci, and mesophilic spore-formers. The attempted isolation of pure cultures of anaerobic cellulose bacteria has attracted the attention of microbiologists for a long time. However, it is only in recent years that unqualified success has been achieved. Many workers have reported the isolation of cellulose-decomposing microorganisms from rumen contents. The reviews of Hungate (9) and of Sijpesteijn (11) have covered extensively the early work on this problem up to 1950. Since that time a number of investigations have been published reporting on improved techniques for isolating cellulolytic bacteria from the rumen.

In early 1951, Gall and Huhtanen (5) reported that about 5,000 isolations of bacteria from the rumen of cattle and sheep had been examined. A description of some of the physiological characteristics of five rumen bacteria was included. In the same year, Sijpesteijn (12) reported that two strains of Ruminococcus flavefaciens were isolated. In the same month Bryant (1) discussed some characteristics of the different bacteria in the rumen of cattle, and later, Huhtanen, Rogers and Gall (8), reported on improved isolation techniques. During the latter half of 1952, Bryant (2) reported the isolation and characterization of an anaerobic spirochete from the bovine rumen. It was found to grow rapidly at 39°C. on media containing rumen liquid, fermentable sugar, and initial pH of 6.5 to 7.0. Recently Bryant and Burkey (3) and Huhtanen and Gall (6,7) discussed cultural methods and characteristics of a number of microorganisms from the rumen of the bovine.

Doetsch, Robinson, and Shaw (4) in 1952, presented data resulting from a critical examination of some of the cultural techniques published earlier on studies of rumen contents. They found that this investigation was necessary since difficulty had been encountered with the methods.

In connection with a program in our laboratories for the study of the intermediary metabolism of ruminal organisms, it became desirable to isolate pure cultures of cellulose-digesting organisms from the rumen. The techniques employed were modifications of those of Hungate (9) and Doetsch et al. (4). The cellulose of the media described by these workers was replaced with carboxymethylcellulose (CMC-70-L) (13). The source material for the isolation was fresh rumen contents, obtained from a

[1]Journal paper No. J - 2368 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project 1202.

[2]Present address, University of British Columbia, Vancouver.

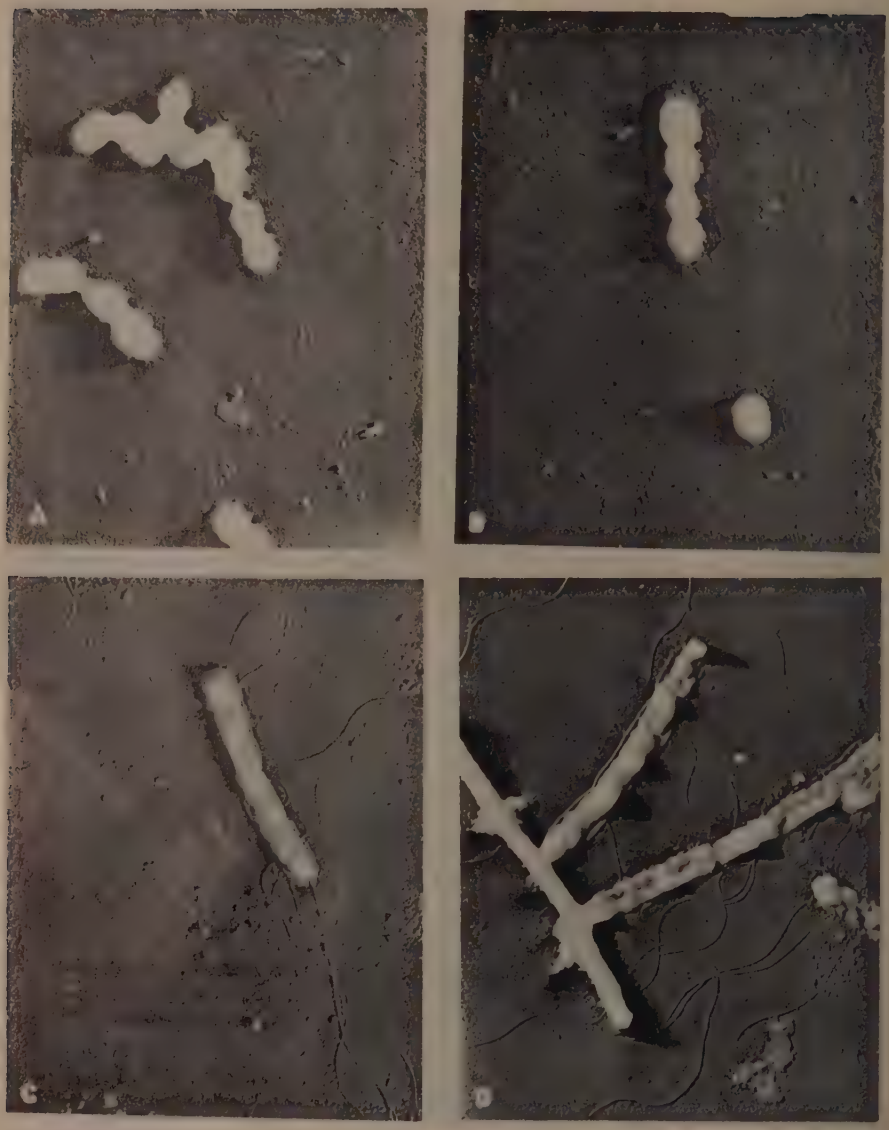

Fig. 1. Electron microphotographs of 24-hour cultures of cellulolytic rumen isolates.

A and B - Culture 31 (magnification 7,500x)
C and D - Culture 32 (magnification 7,500x)

fistulated bovine, strained through four thicknesses of No. 50 cheesecloth to remove suspended solids.

Thirty-two cultures were isolated and the fermentation habits of each were investigated. All 32 cultures fermented starch, dextrin, maltose, cellobiose and glucose. However, only two cultures, designated as 31 and 32, were found to ferment cellulose and CMC-70-L at an active rate.

Microscopic studies revealed that the cells of culture 31 were spherical and nonmotile, occurring singly, in pairs, and in short chains. In cultures of all ages the cells invariably stained Gram positive. The cells of culture 32 were motile rods of varying length. The staining characteristics of culture 32 were variable; in young cultures the cells were Gram negative, while in older cultures they were Gram positive. Both organisms were strict anaerobes. Electron microphotographs of cultures 31 and 32 which had been grown for 24 hours were prepared and are shown in Fig. 1.

The cellulolytic activities of the isolated cultures have been studied further and are reported elsewhere (10).

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THE DEVELOPMENT OF THE EMBRYO OF ZEA IN RELATION TO POSITION ON THE EAR[1]

Max E. Bell[2]

Department of Botany and Plant Pathology, Iowa State College
Ames, Iowa

Studies of normal and abnormal embryology of maize have been based most commonly on samples taken from an arbitrarily selected, but consistent position on the ear. Previous studies have shown that if samples of kernels are taken from a comparable position on different ears, a valid comparison of the status of embryological development can be made between widely dissimilar strains or varieties of corn (2).

In some cases it is necessary to take a sample from an ear, and to permit the rest of the ear to mature on the plant (9). The question arises whether such a sample represents the average condition of embryo development on that ear at the date of sampling. Embryo and kernel size and weight are known to vary on different parts of an ear. Comparisons of embryo development in relation to position on the ear have not been reported. The present study was undertaken to compare organogeny of embryos collected from specific locations on the ear.

REVIEW OF LITERATURE

The developmental morphology of the caryopsis of maize has been described by Randolph (5). In the variety that he examined, the first leaf was initiated between fourteen and sixteen days after pollination. Two leaves were present at twenty days, and five leaves at maturity. He pointed out that five embryonic leaves are usually present in the mature kernel of maize.

Comparative studies of embryo and kernel dimensions and weights have been based largely on samples taken from shelled grain or from arbitrarily designated zones of the ear. Sprague (10) found that embryo weight in hybrids was not consistently correlated with embryo weight of the parental inbreds. Bindloss (1) reported that there was no correlation between weight of embryos and the size of plumular meristems.

Magee (4) compared the organ initiation of inbred and hybrid maize, using leaf initiation as a criterion of development. She found that the influence of the maternal parent was evident in the rate of development of organs. Hybrid vigor in the embryo was expressed by a more rapid rate of leaf initiation.

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[2]The writer wishes to express special appreciation to Dr. John E. Sass who suggested the problem and guided the research. Thanks are also due to Dr. Harry L. Weaver of the University of Nebraska for collecting and processing the material of hybrid 939 used in part of this study.

Fairchild (2) found that eight inbred lines of the corn belt consistently had five embryonic foliage leaves by the fortieth day after pollination, and that the rate of organogeny of hybrids may be less, or greater, than that of the parental inbreds.

Sass (7) found five embryonic leaves in flint and dent varieties, in a very early short line and in a giant Central American variety. A random sample of ten kernels from shelled grain was found to be adequate for a reliable determination of leaf number.

MATERIALS AND METHODS

The types of Zea used in this study were yellow dent, sweet corn and popcorn. The dent types were "stiff-stalked synthetic," the double cross hybrids 939, (L205 x L289) (Os420 x Os426), U. S. 13, (WF9 x 38-11) (Hy x L317), and Hopi corn. The popcorn used was P38, which is a three-way cross, (SG16 x SG32) x SA24. The sweet corn was 3472 x 3515, a single cross, experimental hybrid of the evergreen type.

Pollinations were made by hand, and samples were taken at the desired intervals. The kernels were subdivided and killed in Craf III, dehydrated in a dioxan-normal butyl alcohol series, and embedded in paraffin (6). Longitudinal sections of the younger embryos were best for the study of early organogeny. From twenty to forty days after pollination, observations were made only in the plumule, mostly from transverse sections.

The stiff-stalked synthetic, popcorn, sweet corn, and Hopi Indian corn ears were sampled in the dry mature state, at the positions shown in sodium sulfite and 2 per cent lactic acid. After soaking for twenty-four hours at 7°C. and subsequently for twenty-four hours at 35°C., the embryos were extracted. The plumular region was cut off and killed and embedded in the manner described previously. The brittle, embedded tissues were prepared for sectioning by soaking in warm water (8). The haemalum-safranin stain was satisfactory for most of the observations.

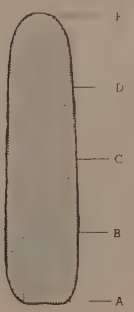

Fig. 1. A diagrammatic representation of the five linear sampling positions used in this study.

OBSERVATIONS

A study of embryo development was made in the maize hybrid U.S. 13, to determine the range of variation of embryo development on an ear at specific dates after pollination. Three ears were collected for each sample and kernels were taken from each of the five positions indicated in Fig. 1.

Organogeny in this hybrid is clearly underway eight days after pollination. At this age, the club-shaped proembryos of U.S. 13 vary considerably in length and diameter. Some proembryos are long and thin, whereas others are short and broad. The greatest variation in embryo length on an eight-day ear was from 27 to 45 microns. Regardless of differences in dimensions, the above proembryos are essentially at the same stage of morphological development with respect to the delimiting of the root-stem axis and the extent of posterior and distal scutellar emergence (Fig. 2). At eight days, the axis is not sharply delimited, but its general outline is indicated by the dense, deeply stained cytoplasm of this zone of meristematic activity. The endosperm occupies approximately one-third of the length of the caryopsis at this age.

Ten days after pollination, the oblique root-stem axis is clearly defined. The stem tip is evident as a large protrusion on the anterior face of the embryo, and the coleoptile is represented by an annular ridge encircling the stem apex (Fig. 3). The first leaf has been initiated opposite the scutellum, on the proximal surface of the stem tip. The surface layer or tunica of the embryo is well defined over the entire surface. The endosperm has almost completely filled the distal region of the kernel.

Embryos from the extreme base and the extreme tip of the ear are somewhat smaller than embryos from other positions. However, organogeny is at essentially the same stage, in the sense that the number of evident organs is the same, although some variation in size of the youngest organs occurs. More variation occurs between embryos from different ears than between embryos from the same ear.

The twelve-day embryos show the same essential uniformity of organ development and narrow range of size as the younger embryos. The greatest variation in embryo length found on any one ear was from 112.5 to 207 microns. Of the sample of 15 twelve-day embryos from three ears, one embryo has the very small primordium of the first leaf, one embryo shows the initiation of the second leaf, but the other thirteen embryos have a uniformly developed first leaf. The coleoptile continues to elongate by activity of the basal annular meristem, and the edges of the coleoptile meet after the twelfth day.

Fourteen days after pollination, the embryos from the different positions on the ear are strikingly uniform with respect to organ development. Two foliage leaves are present, the second usually very small (Fig. 4). The coleoptile edges are nearly united. Although there is some variation in embryo length, from 207 to 247.5 microns, the embryos are much more uniform in size than in the earlier collections.

The sixteen-day embryos were found to be essentially uniform, with two large leaves and the small primordium of the third leaf. The coleoptile edges are completely united. Embryos from the tip and base of the ear are slightly smaller than from other positions.

By eighteen days, the embryos have three well defined leaves. There seems to be no measurable difference in the state of organogeny in embryos from any position on the ear.

The twenty-day kernels still have three leaves which have enlarged by marginal growth, but there is no indication of a fourth leaf. The fifteen embryos of the sample are essentially identical in organogeny (Fig. 5).

The fifth embryonic leaf is evident as a small bulge by the thirtieth

Fig. 2. Longitudinal section of an eight-day embryo. (194x)

Fig. 3. Longitudinal section of a ten-day embryo. (100x)

Fig. 4. Longitudinal section of a fourteen-day embryo. (60x)

Fig. 5. Transverse section of a twenty-day embryo. (100x)

al = anterior lobe
ap = shoot apex
co = coleorhiza
col = coleoptile
dl = distal lobe
lf_1 = first leaf
lf_2 = second leaf
lf_3 = third leaf
lf_4 = fourth leaf
lf_5 = fifth leaf
pl = posterior lobe
ra = radicle
sc = scutellum
su = suspensor


dl
pl
al
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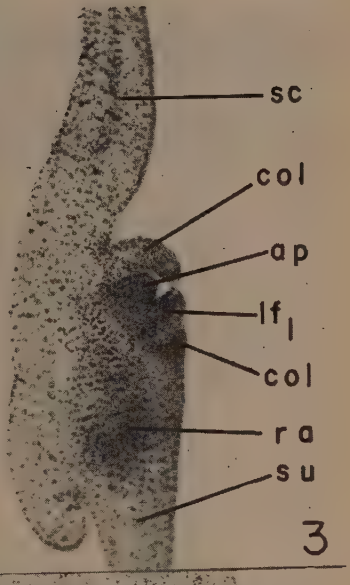
2


sc
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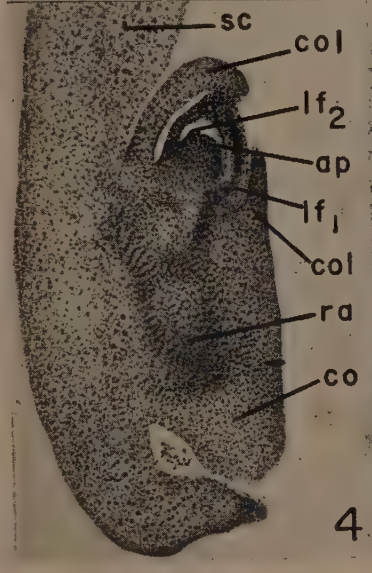
sc
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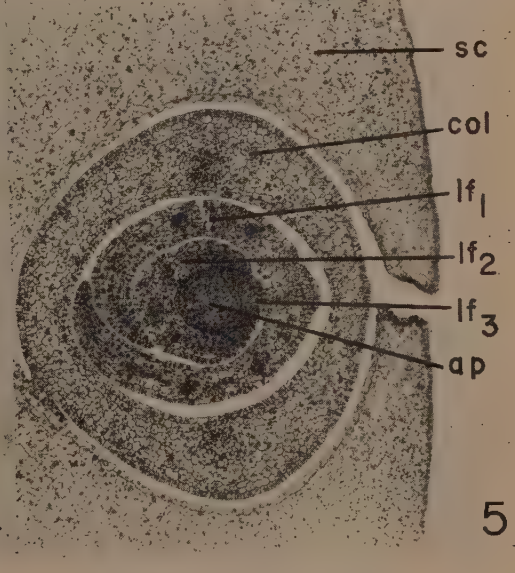
sc
col
lf1
lf2
lf3
ap
5

day after pollination (Fig. 6). Between the thirtieth and fortieth day, the fifth leaf enlarges laterally until it is a crescent that extends part way around the stem in transverse aspect (Fig. 7).

The double cross hybrid, Iowa 939, was used to study the correlation between embryo development on the adaxial, abaxial, right and left side positions on the ear, at the five linear levels, on one date. The twenty samples from each of three ears were handled in the manner described in the materials and methods. Sixty serial cross section slides were obtained at the pertinent levels of the plumular axis. Nineteen days after pollination, one well developed leaf was found in all sixty kernels of the sample, and the coleoptile edges were not completely fused. Organ development of the embryo, therefore, was found to be completely uniform nineteen days after pollination, regardless of position around the ear, or along the length of the ear.

Embryos from the dry mature kernel were studied in a yellow dent (stiff-stalked synthetic), a popcorn (P38), an experimental sweet corn (3472 x 3515), and Hopi Indian corn. Five embryonic leaves are evident in all the serially sectioned embryos. In the popcorn, the fifth leaf is much smaller than in the other lines studied and may be represented only by a zone of meristematic activity just below the stem apex. The embryos of all the lines studied in the dry mature stage were completely uniform with respect to the five foliage leaves that had been laid down.

DISCUSSION

The sampling methods used in studies of maize embryology have been in need of clarification. Some authors make no mention of the position on the ear from which samples were taken. Recent studies have taken into account the possibility of variation in organogeny at different positions on the ear. Magee (3) and Fairchild (2) took samples from an arbitrarily fixed position on hand-pollinated ears. Sass (7) used a random sample of shelled kernels. The present study supports the validity of the foregoing sampling methods, and shows that wide latitude of location is permissible in sampling an ear.

In view of the uniformity of organogeny eight days after pollination, and thereafter to maturity, it is not necessary to section and examine many kernels to obtain a precise diagnosis. A sample of ten kernels is certainly adequate. Of the 480 serially sectioned embryos examined in this study, only two were found to vary even slightly from the rest of their particular sample.

Embryos of a given age vary considerably in length, diameter, and weight, but the present study has shown that the rate of initiation of organs is uniform on an ear. Therefore, dimensions and weights of embryos are not valid criteria of the development of organs and tissues in embryos. Investigations that require a diagnostic standard for evaluating or comparing embryos should make wider use of the status of organogeny, rather than the weight or dimensions of the embryos under investigation.

Fig. 6. Transverse section of a thirty-day embryo. (60x)

Fig. 7. Transverse section of a forty-day embryo. (60x)

ap = shoot apex
col = coleoptile
lf_1 = first leaf
lf_2 = second leaf
lf_3 = third leaf
lf_4 = fourth leaf
lf_5 = fifth leaf

SUMMARY

This investigation was undertaken to explore sampling methods used in embryological studies in maize. Organogeny was used as the criterion of the developmental stage of embryos. Kernels were taken from each of five arbitrary, consistent positions on ears.

Embryos of dry, mature kernels from pop, sweet, and dent varieties had five embryonic leaves, regardless of position of the kernel on the ear.

In hybrid 929, sampled nineteen days after pollination, organogeny was at the same stage in all embryos regardless of linear or circumferential position on the ear.

The hybrid U.S. 13 was sampled at two-day intervals, from eight days to twenty days after pollination, and again at thirty and forty days. Organogeny was uniform at all positions, at all ages, and the ultimate leaf number of five was attained in less than forty days.

Embryo dimensions, and possibly weights, are believed to be unreliable as criteria of the development of organs and tissues in the maize embryo. The status of organogeny of the embryo is probably a more reliable criterion for determining or comparing developmental rates.

In view of the hazards of processing material and preparing slides, a collection of ten kernels from an ear is an adequate sample to provide a reliable diagnosis of organogeny, eight days or more after pollination.

A valid comparison of the organogeny of embryos may be made from kernels collected from any position, or any random combination of positions, on an ear.

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THE RATE OF AMMONIA PRODUCTION IN THE ACID HYDROLYSIS OF VITAMIN B_{12}

John M. Brierly, Robert R. Sealock[1], and Harvey Diehl

Department of Chemistry, Iowa State College, Ames, Iowa

The presence of five amide groups in vitamin B_{12} has been shown by Ellingboe and Diehl (1). These groups account for the five molecules of ammonia released on the acid hydrolysis of the vitamin (2). In the present paper we report a significant difference in the character of these groups as shown by the rate at which ammonia is liberated when the vitamin is digested with hydrochloric acid. Auxiliary experiments were performed to determine the rate of hydrolysis of sodium cyanide for the hydrolysis of cyanide complicates the experiment inasmuch as the B_{12} molecule contains one cyanide group.

EXPERIMENTAL WORK

A solution of 119 mg. of vitamin B_{12} in 50 ml. of water was prepared and by analysis shown to contain 0.439 mg. of cobalt per 4.00 ml. Aliquots of 4.00 ml. were heated with 4.00 ml. of 2 N hydrochloric acid at 95° for specified times in test tubes provided with reflux condensers. The solutions were placed in the water bath with the bath previously heated to 95°. At the end of the digestion period, the tubes were immediately plunged into ice water. They were then kept at 0° until analyzed.

An aqueous solution containing 0.068 mg. of sodium cyanide per ml. was prepared. Five ml. aliquots of this solution were hydrolyzed for various lengths of time in a manner identical with that described above.

Aliquots of the cold solutions resulting from the hydrolysis were transferred to the Parnas micro Kjeldahl apparatus, neutralized by the addition of 4 ml. of 1N sodium hydroxide, buffered to pH 8.5 by the addition of 15 ml. of 0.05 M. phosphate buffer and distilled for 7 minutes. The ammonia was collected and titrated in the usual manner.

RESULTS

The values obtained are given in Table 1. Approximately three moles of ammonia were released within the first thirty minutes of hydrolysis. Approximately four hours were required for the release of the fourth mole and eight hours for the fifth. In twenty hours a total of 5.62 moles of ammonia was liberated, the excess above five being derived from the cyanide group.

The hydrolysis of sodium cyanide for 30 minutes failed to yield any ammonia. In a 20-hour hydrolysis only 36 per cent of the theoretical amount of ammonia was formed.

[1]Deceased, August 19, 1951.

Although the hydrolysis of the cyanide of sodium cyanide and of B_{12} quite possibly do not proceed by the same mechanism, it seems reasonable to conclude that the first three, rapidly formed ammonia are derived from the amide groups of B_{12} and that the three amide groups yielding this ammonia are much more readily hydrolyzed than the remaining two. The remaining two also differ significantly from each other in the ease of hydrolysis.

The infrared spectrum of B_{12} shows no absorption band between 5.0 and 6.0 μ and for this reason lactam rings are probably not present in the molecule. The difference in the ease of hydrolysis must then arise from steric factors.

TABLE 1

Rate of production of ammonia during the hydrolysis of vitamin B_{12}. 1 N hydrochlorich acid, 95°C.

Time hours	Volume HCl 0.0102 N ml.	Nitrogen mg.	Ratio N:Co
0.25	1.80	0.256	2.59
0.50	2.20	0.313	3.17
1.00	2.25	0.320	3.24
2.00	2.47	0.351	3.56
4.00	3.05	0.433	4.40
8.00	3.28	0.466	4.72
20.00	3.70	0.542	5.62

ACKNOWLEDGEMENT

The authors wish to express their appreciation to the Squibb Institute for Medical Research for generous grants of funds and materials which have made possible the work on vitamin B_{12}, of which the present work was a part.

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EFFECTS OF HEATING VITAMIN B_{12} IN A STREAM OF DRY NITROGEN AT VARIOUS TEMPERATURES

Richard Murie and Harvey Diehl

Department of Chemistry, Iowa State College,
Ames, Iowa

The presence of five amide groups in the molecule of vitamin B_{12} was shown by Ellingboe and Diehl (1). Various experiments with vitamin B_{12} and its red, acidic hydrolysis product lead us to believe that at least some of these acid amide groups are located sufficiently closely to permit the formation of cyclic anhydrides or imides. The experiments being reported here were designed to determine if vitamin B_{12} could be converted directly to an imide by expulsion of ammonia by direct heating.

Vitamin B_{12} has been dried at temperatures up to 100°, apparently without detectable decomposition (2). It is reported to darken without melting at 190 to 250° (3).

EXPERIMENTAL WORK

Commercial cylinder nitrogen was passed through a vanadous sulfate train to remove oxygen, and then successively through tubes containing calcium chloride, ascarite, and anhydrous magnesium perchlorate to remove water, ammonia and acidic gases.

The vitamin B_{12}, in a small platinum boat, was placed in a glass tube surrounded by a cylindrical, electrically heated jacket. The glass tube was then connected to the nitrogen train and on the outlet side to an absorption vessel. Connection was made through ground glass joints secured by wire springs. For weighing, the tube was closed by other ground glass joints bearing short lengths of capillary tubing.

A weighed quantity of vitamin B_{12} was placed in the boat, and the train assembled. A measured volume of standard hydrochloric acid was placed in the absorption vessel. The train was swept with nitrogen and the temperature was adjusted. After the heating periods, the system was allowed to cool to room temperature, the nitrogen stream discontinued, and the tube and boat weighed and the acid titrated with standard base. Thus, the loss in weight was obtained as well as the ammonia liberated.

The standard solutions used were approximately 0.002 N in concentration. The titrations were carried out potentiometrically, precautions being observed to avoid the introduction of carbon dioxide from the atmosphere.

Following these operations the remaining material was ground with Nujol and its infra-red absorption curve obtained on the Baird recording spectrophotometer. The Nujol mull was then treated with benzene and ether and the vitamin B_{12} residue dissolved in water. The ultraviolet and visible absorption of this solution was then obtained using a Beckman DU spectrophotometer.

Experiment 1

The sample of vitamin B_{12} was held for eight hours successively a each of five temperatures from 109° to 210°; Table 1.

The 210° product, a black material, was soluble in water yielding a brown-orange solution. This solution turned purple when treated with an excess of sodium cyanide.

The infrared spectrum of the 210° product showed some modification in the bands at 6.0 and 6.2 μ and a new band at 5.7 μ.

Experiment 2

The sample was held for 20 to 24 hours at each successive temperature, the final temperature being 243°; Table 1.

The 243° product, black in color, was not soluble in water, benzene, methanol, dioxane, acetone, carbon disulfide or chloroform. It yielded a brown solution when dissolved in a sodium cyanide solution.

The infrared spectrum of the 240° material showed a new band at 5.7 μ and some modification in the bands at 6.0 and 6.2.

TABLE 1

Loss in weight and ammonia liberated on heating vitamin B_{12}

Temp. °C.	Initial weight mg.	Final weight mg.	Change in weight mg.	Change in weight per cent	Ammonia liberated mg.	Ammonia liberated meq.	Mole ratio * NH_3/B_{12}
Experiment 1							
109	22.280	19.775	-2.505	-11.23	0.00	-	-
123	19.775	20.058	+0.283	+ 1.27	0.00	-	-
155	20.058	19.904	-0.154	- 0.69	0.032	0.00191	0.135
186	19.904	19.437	-0.467	- 2.09	0.033	0.00191	0.135
210	19.437	18.828	-0.609	- 2.73	0.088	0.00517	0.368
Total			-3.452	-15.47	0.153	0.00899	0.638
Experiment 2							
100	22.607	19.098	-3.509	-15.470	0.0169	0.00100	0.0805
123	19.098	18.998	-0.100	0.442	0.0101	0.00060	0.0482
143	18.998	18.808	-0.190	0.839	0.0169	0.00100	0.0805
183	18.808	17.953	-0.855	3.780	0.0777	0.00457	0.3630
210	17.953	17.215	-0.738	3.260	0.1500	0.00886	0.7090
Total			-5.392	23.791	0.2716	0.0160	1.22
243	17.215	16.213	-1.002	4.320	0.0948	0.00558	0.4440
Total			-6.394	28.1	0.366	0.02161	1.7252
Experiment 3							
175-80	59.855	48.047	-11.808	19.75	0.5955	0.0350	0.965

*Milliequiv. B_{12} = (Final wt. + wt. NH_3 liberated)/1340.

Experiment 3

The sample was heated for 14 hours at 100° and then for five days at 175 to 180°; Table 1.

CONCLUSIONS

At 180°, in an atmosphere of nitrogen, one molecule of ammonia is expelled from vitamin B_{12}; the process is accompanied by a much greater loss in weight than would be expected for the expulsion of this ammonia alone, 19 per cent instead of 1.0 per cent. The additional loss may be merely due to loss of water inasmuch as the ultraviolet and infrared absorption spectra are not much changed. The peak at 361 μ was shifted to 352 μ and a new band appeared in the infrared at 5.7 μ. The latter would be expected ao appear as the result of cyclic imide or anhydride formation. At 240° two molecules of ammonia are expelled but the other changes in the molecule are much more extensive.

ACKNOWLEDGEMENT

The authors wish to express their appreciation to the Squibb Institute for Medical Research for supporting their work on vitamin B_{12}.

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EFFECTS OF NATURAL SELECTION IN SEGREGATING GENERATIONS UPON BULK POPULATIONS OF BARLEY[1]

L. H. Taylor and R. E. Atkins[2]

In recent years, large acreages of small grains in certain areas have been planted to one variety or to a few varieties of similar parentage. The presence of such quantities of similar host material provides an ideal situation for rapid build-up of parasitic diseases. A severe reduction in yield and quality of the crop has resulted in some seasons. As future disease problems are somewhat unpredictable, investigators have seen the need for a wider base in the germ plasm of small grain varieties. Fortunately, large and diverse collections of small grain strains have been made and these, with continued plant introductions, provide many new sources of disease resistance for incorporation into breeding programs.

The bulk method of breeding small grain seems best adapted to the observation and evaluation in early segregating generations of large numbers of crosses. It generally is assumed by those using the bulk method that high yielding and disease resistant plants will survive and in each successive generation constitute a larger proportion of the bulk population. Superior germ plasm should thus be available for subsequent selection and evaluation as pure lines. The validity of this assumption may be questioned on the basis of data from several studies.

Relatively little is known of changes in gene frequency for specific characters due to natural selection within bulk populations in segregating generations following a cross. The major objective of the investigations reported herein was to study changes in bulked hybrid populations of barley subjected to different environmental conditions in four areas of Iowa. Changes in such populations brought about by natural selection may have fundamental as well as practical significance.

REVIEW OF LITERATURE

In the improvement of self-pollinated cereals, plant breeders have used hybridization followed either by the bulk, the pedigree, or a combination of the two methods of handling segregating populations. The essential features and procedures of each method have been outlined by Hayes and Immer (11) and by Love (15).

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[1]Contribution from the Agronomy Department, Iowa Agricultural Experiment Station, Ames, Iowa. Journal paper No. J-2403. Project 1177. Part of a thesis submitted by the senior author to the Graduate Faculty of Iowa State College in partial fulfillment of the requirements for the Ph. D. degree.

[2]Formerly Graduate Assistant in Farm Crops, Iowa Agricultural Experiment Station, now Assistant Professor in Agronomy, Maine Agricultural Experiment Station, Orono, Maine, and Associate Professor of Farm Crops, Iowa Agricultural Experiment Station. The authors are indebted to Drs. I.J. Johnson, M.G. Weiss, and D.D. Morey for making the original crosses and assistance in other early phases of the study.

One of the advantages attributed to the pedigree system is that it permits a joint attack on breeding and inheritance problems. The bulk system has the advantage of requiring less detailed work in early segregating generations, which permits evaluation of more crosses and growing a larger sample of segregating populations.

Yield tests to predict the value of bulk and segregated populations have been studied by several investigators. Harrington (10) found yields in replicated bulk F_2 trials to be valuable in predicting the subsequent yield performance of F_6, F_7, and F_8 selections from six wheat crosses.

Yield trials in F_3 were considered to be of supplementary value. The bulk population method also was used effectively with wheat crosses by Florell (5). He suggested this method should be particularly valuable in breeding for winter hardiness and for rust and smut resistance in the cereals.

In an extensive study with barley, Harlan, Martini, and Stevens (9) concluded that yields of bulked populations were a good indication of the crosses from which high-yielding selections could be obtained. Low-yielding crosses could have been discarded on the basis of their bulk yields without appreciable loss. Immer (12) compared the bulk F_2, F_3, and F_4 generations of six barley crosses in replicated yield trials. Two crosses that produced the highest yields in F_2 and F_3 were also among the highest in F_4, while two other crosses were relatively low-yielding in all generations tested. It was concluded that bulk yield trials could be used to advantage in discarding certain crosses. Suneson and Riddle (19) studied hybrid vigor in barley and proposed a method of evaluating the yield-transmitting qualities of varieties through tests of their F_1 hybrids.

Results presented by other investigators do not confirm the belief that bulk population yield data give valuable information on the yield potential of crosses. With soybeans, Weiss, Weber, and Kalton (22) found that bulk population tests of the F_2 through F_5 generations gave adequate evaluation of the performance of subsequent selections for plant height and lodging resistance but not for grain yield or maturity date. Atkins and Murphy (3) studied ten bulk hybrid populations of oats. Those which gave highest yields in segregating generations did not subsequently produce the greatest proportion of high-yielding segregates. From a study of early generation bulked progenies of barley, Grafius, Nelson, and Dirks (7) concluded that if early generation testing of bulked hybrids is to be accompanied by selection of entire crosses on the basis of new characters, controlled by relatively few epistatic genes, then such a program might be worthwhile. Otherwise they believed the mean of the two parents involved should give a better estimate of the heritable variance than an early generation bulk progeny test. They further pointed out that on this basis it would appear that the between cross early generation testing of bulked self-pollinating small grains is of little value insofar as breeding for yield is concerned.

Lambert* related the yield of F_5 lines from ten barley crosses having Mars as a common parent with their performance in bulk F_2 and F_3 yield tests. He found little encouragement for using bulk tests to evaluate the

*Unpublished data from J. W. Lambert, Department of Agronomy, University of Minnesota, St. Paul, Minnesota.

potentialities of crosses for production of high-yielding lines. The parental means appeared to be a somewhat better index of the potentialities of a cross. Similarly, Fowler and Heyne (6) grew forty-five crosses among ten varieties of wheat in bulk through the F_5 generation and tested an equal number of selections from each cross. Analysis of yield data from the early generations indicated that bulk yield tests were of little predictive value.

The effects of natural selection and competition in changing the composition of bulk populations made up by artificially mixing equal quantities of small grain varieties have been investigated by several workers. A mixture of equal amounts of eleven barley varieties was made by Harlan and Martini (8) and grown at ten experiment stations under a wide range of conditions for periods of from four to twelve years. The dominant variety at each station soon was evident, and varieties that were dominant at one or more stations often were eliminated at others. At some stations the variety that became predominant was not considered particularly well adapted to the area, and varieties that were widely grown commercially tended to be eliminated from the mixture.

Klages (13) grew a mixture of durum and hard red spring wheat for a single season. A very large increase in the rust-resistant durum component of the mixture was explained by the occurrence of a severe stem rust epiphytotic. Laude and Swanson (14) mixed equal numbers of seeds of the winter wheat varieties, Kanred, Harvest Queen, and Currell, and grew the mixture at two locations in Kansas over a nine-year period. Shifts in the varietal ratios resulted in nearly pure stands of Kanred, the more adapted variety, by the end of the experiment. Changes in the varietal ratio were attributed both to competition among plants which resulted in greater survival of the more adapted variety and to production of more seeds per plant by Kanred.

Two different barley varietal mixtures and a mixture of five wheat varieties were grown by Suneson and Wiebe (20) for periods of five to nine years. They found that the survival of varieties in a mixture bore no relationship to the yields of component varieties when grown in pure stands. Vaughn barley and Ramona wheat were well adapted and high-yielding when grown in pure stands but proved to be very poor competitors in the mixtures. These data suggested a decided limitation to the bulk method of breeding because plants that survive best in the hybrid mixture may not be the best types when grown alone. This study was continued by Suneson (18) who grew a mixture of four barley varieties for sixteen years. At the end of this time the proportions of varieties in the mixture were Atlas 88.0 per cent, Club Mariout 10.5 per cent, Hero 0.7 per cent, and Vaughn 0.4 per cent. During this period, Vaughn yielded significantly higher than any of the other varieties when tested in pure stands and Hero was second in yield. Vaughn and Hero also were considerably more resistant to the leaf diseases which were serious in some years of the study. Suneson suggested, on the basis of these results, that the bulk method of breeding may not necessarily perpetuate either the highest yielding or the most resistant progenies but rather those with an intangible character of competitive ability.

Another factor that may affect yields and survival in a mixture is the weight per kernel of seed planted. Waldron (21) separated seed of a pure

line of wheat into two classes on the basis of seed weight. Subsequent yield tests using either the same number of kernels per unit area or the same weight of seed showed that the heavier kernels gave significantly higher yields. Heavy greenhouse-produced seed also was found to give significantly higher yields than lighter seed of the same strains produced under field conditions.

Information is limited on the effects of natural selection in hybrid populations on changes in gene frequency for specific characters in small grains. In six oat crosses, grown as bulk populations until F_7 or F_8, Atkins (2) found natural selection during this period had been very effective in increasing the proportion of types resistant to prevalent races of crown and stem rust, and to Helminthosporium victoriae.

Three bulk hybrid populations of rice were grown by Adair and Jones (1) for eight generations at locations in Arkansas, Texas, and California. Each population was a composite of seed in the F_2 of from three to eleven crosses, the original composition of the composites being somewhat different for the three locations. A study of the types that survived in the bulk populations showed selection for heading date and plant height had differed with location. The proportions of different grain types and of awnless plants that survived also were quite different.

Studies on the segregation of a composite barley hybrid were made by Middleton and Chapman (16) for the F_3 to F_5 generations at two locations in North Carolina. They found natural selection favored rough over smooth awns and six-row over two-rowed types. Selection favored awned over hooded types at one location but not at the other.

EXPERIMENTAL PROCEDURE

Hybrid populations of 20 barley crosses were used in the study reported herein. The original crosses had been previously made during the winter of 1944-45. The F_1 generation was grown at Aberdeen, Idaho, in 1945, and the F_2 and subsequent generations as part of the breeding program at the Iowa Station. In 1947, these 20 crosses were selected from a larger group largely on the basis of remnant F_1 seed available. Parentage of the crosses is given in Table 1.

In most of the crosses, at least one parent was agronomically desirable in Iowa, and the other had exhibited some disease resistance during the poor barley season of 1944. Khayyam and Tennessee Winter are winter barleys, and four crosses with them – H1, H5, H30, and H69 – were hybrids of winter x spring types. Horsford is a hooded barley, and H25, H42, H44, and H46 were crosses of hooded x awned types. Khayyam is a two-rowed barley, and H1, H5, and H30 were crosses of 2-rowed x 6-rowed types. Ten of the crosses were of smooth-awned x rough-awned types, and the crosses as a group represented a wide diversity of germ plasm.

The 20 crosses in F_2, F_3, and F_4 were grown at four locations chosen to represent a diversity of climatic conditions for barley in Iowa. These locations were near Shenandoah, Ames, Marcus, and Cresco. Shenandoah, in southwestern Iowa, and Ames, in central Iowa, are in areas where climatic conditions in most seasons might favor selection for a different type of barley than at Marcus in the northwestern and Cresco in

TABLE 1

Parentage and hybrid number of bulk hybrid barley populations

Hybrid No.	Cross
H1	Khayyam x Glabron-Peatland Sel. 53
H5	Khayyam x Wis. 38-Chevron Sel. 75
H13	Manchuria 4471 x Velvet-Peatland Sel. 31
H16	Cape x Mars
H19	Coast x Peatland
H21	Coast x Manchuria 4471
H25	Horsford x Chevron
H29	Cape x Manchuria 4471
H30	Khayyam x Peatland
H37	C.I. 5075 x Velvet-Peatland Sel. 31
H40	C.I. 5075 x Coast
H42	Horsford x Scotch
H44	Horsford x Wis. 38-Chevron Sel. 75
H46	Horsford x Glabron-Peatland Sel. 53
H52	C.I. 3235 x Wis. 38
H53	Scotch x Velvet-Peatland Sel. 31
H60	Scotch x Glabron-Peatland Sel. 53
H69	Tennessee Winter x Glabron-Peatland Sel. 53
H71	Cape x Peatland
H110	Quinn x Wis. 38-Chevron Sel. 75

the northeastern section of the state. The two northern locations are in areas where some commercial barley is grown and conditions are considered somewhat more favorable for barley production.

In 1947, at each of the four locations, bulk F_2 populations of the 20 crosses, together with five of the better adapted parents, were grown in a 5 x 5 simple lattice design. Two replicates of single rod-row plots were grown at each location. In 1948 and 1949, similar F_3 and F_4 bulk population tests were grown at the four locations, in every case from seed produced at that location in the previous generation. In 1948, grasshopper damage to the F_3 test at Shenandoah was so extensive that the test was abandoned and the F_3 test at Shenandoah repeated in 1949. Thus, seed of only two segregating generations of the crosses was available from Shenandoah.

Remnant seed from all bulk population tests from the four locations was kept at Ames under cold storage to maintain germination percentage at a high level. In order to have F_2 seed for additional tests the crosses were remade during the winter of 1948-49 and the F_1 generation was grown at Aberdeen, Idaho, in 1949.

To more accurately evaluate location effects upon the bulk barley populations in the early segregating generations, seed from all locations for all generations was grown in bulk hybrid tests in 1950 at Ames and Cresco. Entries in each test consisted of the F_3 and F_4 generations of the 20 crosses from Ames, Cresco, Marcus, and Shenandoah, and the F_5 generation from Ames, Cresco, and Marcus. The 20 F_2 populations as well as the 15 spring parents were included using seed produced at Aberdeen, Idaho. The variety Moore also was included in the test to make a total of 256 entries which were planted in a 16 x 16 triple lattice with three replicates at each location. Plots consisted of a single rod-row with alternate rows of Mars barley to provide uniform competition.

Field notes taken on the two tests included maturity (at Ames only) and plant height. Maturity was recorded as the number of days after June 30 that 95 per cent of the heads in a plot were mature. Average height was measured in inches from the ground to the tip of the head, not including awns, and grain yield measured to the nearest gram. Spaced populations of all generations also were grown at Ames to determine the effects of natural selection in differing environments on changes in genetic composition of bulk populations as measured by changes in frequency of several easily classified morphological characters. Only data on maturity, plant height, and grain yield will be presented in this paper.

For the purpose of determining the association of bulk population yields with performance of selections from those bulk hybrids, approximately 50 heads were selected from each F_3 bulk population at Ames in 1948. These were grown as F_4 lines at Ames in 1949 and ten of the most desirable lines selected by visual evaluation for lodging resistance, freedom from disease, and apparent yielding ability. A total of 200 selections from the 20 crosses together with the 20 F_2 bulk populations and five agronomically desirable parents were grown in a 15 x 15 simple lattice design at both Ames and Cresco in 1950. A plot consisted of a single eight-foot row and two replicates were grown at each location. Alternate rows of Mars barley were sown to provide uniform competition.

Methods outlines by Snedecor (17) and by Cochran and Cox (4) were used in analysis of the data. Some difficulty was encountered in evaluating the 1950 rod-row test for differences in segregating generations due to location and generation effects since there was no F_5 generation from Shenandoah. In order to make valid comparisons and make use of all of the data, two different analyses of the segregating generations were made. In one analysis, the F_3 and F_4 generations from four locations were included, while in the other, three segregating generations were used, but only data from Ames, Marcus, and Cresco were included. Hereafter, the first analysis will be referred to as the F_3-F_4 analysis and the latter as the ACM comparison.

EXPERIMENTAL RESULTS

Bulk Population Tests

Grain yield

Variance analyses of the F_2-F_4 bulk population yield tests, grown at the various locations during the period 1947-49, showed yield differences among the 20 crosses were highly significant in all 11 tests. Combined

analysis of the data indicates that in each year there was a highly significant difference among locations for yield and also a highly significant crosses x locations interaction in each of the three years.

Correlations of yield between generations within locations, as shown in Table 2, generally were relatively high, except for the Marcus tests.

Analyses of the bulk population tests of all generations grown at Ames and Cresco in 1950, and examination of the comparative yield rank of different hybrids during the period 1947-49 again indicated a relatively close agreement in performance of hybrids in the two comparisons. Crosses that yielded well in 1950 had ranked relatively high in average yield in bulk tests during the 1947-49 period, while other crosses yielded poorly in both instances. Similarly, yields obtained in the Ames and Cresco bulk hybrid tests in 1950 were generally in good agreement. The correlation coefficient of .606 (218 df) for association of yields in the two tests were highly significant.

TABLE 2

Coefficients of correlation for yield of 20 bulk hybrid barley populations grown at four locations in Iowa, 1947-1949

Generations correlated	Location[1]			
	Ames	Cresco	Marcus	Shenandoah
$F_2 - F_3$	.491*	.847**	.014	.570**
$F_2 - F_4$	.643**	.823**	.266	
$F_3 - F_4$	.564**	.787**	.268	

[1] Eighteen degrees of freedom at each location.
*Significant at 5 per cent level.
**Significant at 1 per cent level.

To compare differences among bulk populations during the early segregating generations, when grown at varying locations, the partition of variance presented in Table 3 was made. Both the analysis using all generations at the three locations and the analysis which included only the F_3 and F_4 generations at all locations are shown and were essentially alike. As the gain in efficiency through use of the lattice design was small, yields were not adjusted. Significant differences among parents for yield were obtained in both tests. Parental yields were significantly higher than the yields of the crosses at Ames but not in the Cresco test. The F_2's differed significantly in yield in both tests, and the F_2 yielded significantly more than the other generations in both tests.

The magnitude of the F_2 versus other generations mean square was large at both Ames and Cresco. Although F_2 yields might be expected to be higher because of greater heterozygosity, it seems probable that factors of seed quality also were of importance in the differences observed. Seed for the F_2 and parents in these tests was produced under irrigation at Aberdeen, Idaho. It was of good quality with plump, heavy kernels and almost completely free from seed-borne diseases. Because seed

TABLE 3

Analysis of variance of yield in grams per plot for the various components comprising varieties and hybrids previously grown in early generations at four locations and tested in 1950 at Ames and Cresco

Source of variation	Degrees of freedom		Mean squares			
			Ames bulk hybrid test		Cresco bulk hybrid test	
	ACM	F_3F_4	ACM	F_3F_4	ACM	F_3F_4
Varieties and hybrids	215	195	7,927**	9,118**	7,310**	9,049**
Among parents	15	15	13,669**	13,669**	10,826**	10,836**
Parents vs. crosses	1	1	7,691*	14,472**	262	1,440
Among F_2's	19	19	11,337**	11,337**	7,225**	7,225**
F_2 vs. other generations	1	1	153,365**	189,091**	176,473**	239,741**
Generations	2	1	3,563	1,556	10,101	14,366*
Locations	2	3	18,230**	47,827**	18,700**	81,657**
Gen. x locations	4	3	2,315	4,323	6,869	7,351
Crosses	19	19	41,582**	39,329**	28,009**	28,408**
Gen. x crosses	38	19	1,602	2,105	2,570	2.893
Loc. x crosses	38	57	2,084	1,546	3,650	2,934
Gen. x loc. x crosses	76	57	1,840	2,115	3,179	3,161
Randomized block error	510	510	1,727	1,727	2,322	2,322

* Significant at 5 per cent level.

** Significant at 1 per cent level.

produced in Iowa was not of comparable quality, all comparisons of parent and F_2 yields with F_3, F_4, and F_5 yields may have been confounded with differences in seed quality.

Significance of differences among generations, locations, crosses or their interactions are dependent upon the assumptions which one wishes to test. For these data it was assumed that the crosses under investigation represented a sample of crosses from a barley breeding program and, thus, that the results here reported might be applicable to groups of crosses other than those included in these tests. The generations involved were the F_2, F_3, and F_4 grown at four different locations chosen to represent a diversity of environmental conditions. The data were interpreted and considered pertinent only to these particular early generations rather than to a random sampling of all possible segeegating generations. Also, the locations tested were chosen to provide environmental differences, and the data thus are applicable only where considerable environmental differences are present rather than to a random sampling of locations.

When tested with the appropriate error term, none of the interactions of generations x locations, generations x crosses, or locations x crosses, shown in Table 3, was significant. Variance among crosses exceeded the 1 per cent level of probability in all partitions while generations differed significantly only for the F_3-F_4 breakdown at Cresco. The effect of location was of particular interest and was highly significant in both divisions of the data and in both the Cresco and Ames bulk population tests. It is apparent that the location at which the segregating bulk populations were grown exerted considerable influence on their subsequent yield performance.

The consistency and magnitude of the effect of location on yield in the two tests is shown in Table 4.

TABLE 4

Mean yield in grams per plot and yield rank of bulk hybrid tests grown at Ames and Cresco in 1950

Location where seed was produced	Yield in 1950 at		Yield rank (Both 1950 tests)
	Ames	Cresco	
Ames	333.45	373.16	1
Marcus	324.83	360.86	2
Cresco	313,39	352.93	3
Shenandoah	291.18	316,90	4

The rank in yield for seed produced at the four locations was the same in both the Ames and Cresco tests and the size of yield differences also was comparable at the two locations. Seed grown during the segregating generations at Ames gave highest yields at both locations in the 1950 tests, with Marcus-produced seed ranking second, Cresco third, and Shenandoah fourth.

From examination of the yield data presented in Table 5, it is apparent that most of the yield advantage of the Ames produced seed can be attributed to the F_4 generation, which yielded significantly more than the average yield at both locations in 1950. Yields of the F_3 and F_4 generations from Shenandoah, however, were significantly below the test means in both 1950 experiments. Neither the seed produced at Marcus or Cresco nor the F_3 or F_5 generations from Ames was significantly higher or lower than the mean yields of the tests.

Further explanation of differences in yield attributable to the varying locations and generations was obtained from kernel weights of seed sown in the 1950 bulk population tests at Ames and Cresco. Using remnant seed, the weight of two random samples of 100 kernels from each entry was measured to the nearest .01 gram.

Analysis of the seed weight data showed the major source of variation to be attributable to the wide difference in plumpness of the parental and F_2 seed produced at Aberdeen, Idaho, as compared with the seed produced in Iowa which was used for planting the F_3, F_4, and F_5 generations. Among the generations produced at the Iowa locations the seed used for growing the F_4 was significantly higher in seed weight than either the F_3 or F_5 seed, which is in agreement with yield data previously cited for these generations. Magnitude of the mean square for location differences was much greater for the F_3-F_4 analysis, as it included the Shenandoah-produced seed which was considerably lighter than seed produced at the other locations. Rankings for weight of seed sown were identical with subsequent yield rankings of seed produced at the four locations with the Ames seed being heaviest, Marcus seed second, Cresco third, and the Shenandoah seed lightest in kernel weight.

The correlation between grain yield and weight of seed sown was determined for the F_3-F_5 generations for both tests in 1950. Coefficients of .290 at Ames and .319 at Cresco (218 df) were significant at the 1 per cent level, but were of only moderate magnitude. This indicated that weight of seed sown had an appreciable influence on subsequent grain yields, but that other factors also were operative in determining yield.

Plant height

Variance among the varieties and hybrids for plant height was analyzed and interpreted as outlined for grain yield. Highly significant differences for height were obtained among parents and among F_2's in both tests. In only the F_3-F_4 partition of the Ames test was there a significant difference between parents and hybrids in height, the hybrids in this case being significantly taller than their parents.

Differences among crosses in plant height were highly significant in both tests. Location effects, however, were not significant, indicating no great differences in selection for plant height at the four environments. In the ACM partition of variance for both tests a highly significant difference among generations was observed. As the difference between generations in the F_3-F_4 partition was negligible, it seemed that the inclusion of the F_5 data was responsible for the large generation effect in the ACM partition. Examination of the data in Table 5 confirmed this assumption, as the F_3 and F_4 populations were found to have almost identical average heights, while the F_5 populations were consistently about one-half inch

TABLE 5

Average grain yield, plant height, and maturity of seed produced at four locations and grown at Ames and Cresco in 1950

Seed source		Yield in grams		Height in inches		Maturity, days after June 30,
Location	Generation	Ames	Cresco	Ames	Cresco	Ames
Ames	F_3	331.10	361.67	36.28	34.47	17.72
	F_4	343.61	395.90	36.58	34.95	17.70
	F_5	325.63	361.92	35.30	34.23	17.38
Cresco	F_3	316.10	349.80	36.30	34.85	18.13
	F_4	312.98	355.08	36.20	34.70	18.43
	F_5	311.08	353.92	35.87	34.40	17.78
Marcus	F_3	332.40	361.37	36.38	34.60	17.88
	F_4	322.57	361.88	36.38	34.83	18.07
	F_5	319.53	359.32	35.70	34.42	17.98
Shenandoah	F_3	283.73	315.03	36.23	34.73	18.02
	F_4	298.62	318.77	35.93	34.08	17.25
Mean of test		324.10	359.89	36.11	34.56	17.75
L.S.D. (5 per cent level)		14.91	17.33	.59	.43	.36

shorter in height. In the F_3-F_4 partition of the Cresco test, a highly significant generation x locations interaction indicated selection for height differences for these two generations was not consistent over the four locations, but this was not borne out in the ACM analysis nor in either of the analyses of Ames data. Similarly, the interactions of generations x crosses and locations x crosses were not significant in either the Ames or Cresco test.

Plant height was significantly correlated with grain yield in both tests. Correlation coefficients of .231 at Ames and .250 at Cresco (218 df) were not of sufficient magnitude, however, to preclude the possibility of obtaining high-yielding short-strawned lines from the bulk populations.

Maturity

Maturity rating of a segregating bulk population is of necessity an approximation. Wide differences in maturity are discernible, however, and the analysis of the Ames test indicated that the differences among varieties and hybrids tested exceeded the 1 per cent level of probability. A further partitioning of the variance for maturity of varieties and hybrids showed that differences among parents and among F_2's were highly significant and that the parents averaged significantly earlier than their hybrids. Differences in maturity between the F_2 and subsequent generations were not significant, but the variance among the F_3-F_5 generations exceeded the 5 per cent level of probability in the ACM analysis, reflecting a trend toward selection for early maturity in the F_5 populations.

Location effects were highly significant in both analyses. Mean maturity dates given in Table 5 indicate these were the result of greater selection for early maturity at Ames and Shenandoah than at the two locations in northern Iowa. A highly significant mean square for the generation x location interaction in the F_3-F_4 analysis indicated that the locations had a differential selection for earliness from F_3 to F_4. Again, reference to Table 5 shows that from F_3 to F_4 the populations grown at Ames were almost constant in average maturity, while Cresco- and Marcus-produced lines shifted slightly toward later-maturing types, and Shenandoah-grown lines showed a marked shift toward earlier maturity.

Grain yield and maturity date were observed to be negatively correlated in the Ames test with a coefficient of -.364 (218 df) which exceeded the 1 per cent level of probability. Maturity date also was significantly correlated with plant height in the Ames test, but the coefficient of -.179 was very low.

Selection Tests

The degree of association between yields in bulk populations and yields of selections made from them was pertinent to this investigation. It should be remembered that the selections in these tests were not homozygous lines, but were F_5 types resulting from head selections made from the F_3 bulk hybrid test at Ames in 1948. They were evaluated in the field as F_4 populations at Ames in 1949, and the most promising were harvested in bulk to provide seed for planting the selection tests. An average of ten selections from each of the twenty crosses was grown at both Ames and Cresco in 1950.

Analyses of variance for yield of the two tests showed highly significant mean squares for the comparison of parents versus hybrids, among F_2's, F_2 versus F_5 selections, among crosses, and within crosses. Differences in yield between parents and hybrids and between F_2 populations and F_5 selections again were undoubtedly influenced by differences in seed quality as the parental and F_2 seed was produced under irrigation at Aberdeen, Idaho, while the other entries were grown from seed produced in Iowa.

A comparison of the yield of selections from the 20 crosses with relation to the yield performance of the bulk populations is presented in Table 6. Two frequency distributions are presented, the selections being grouped into four yield levels according to the order of bulk F_2 yields in one distribution, and in accordance with the order of average bulk population yields from F_2 through F_5 in the other.

Distributions were similar for both groupings, with hybrids that were high-yielding in bulk populations also producing a large number of high-yielding selections. For the distribution based on bulk F_2 yields, the five highest-yielding crosses were markedly superior in number of high-yielding selections with 25 selections exceeding the check varieties, while the five lowest-yielding crosses had only one selection that yielded above the mean of the five parents. When the distribution was based on the average of the F_2-F_5 bulk populations, 20 selections from the five high-yielding bulk populations and 12 selections from the second high group were above the mean of the checks, whereas with the five lowest-yielding bulks there were no selections superior in yield to the check mean.

TABLE 6

Frequency distribution of selections from 20 barley crosses grown at Ames and Cresco in 1950, based on units of standard error of the difference above or below the mean of five check varieties*

Grouped according to order of bulk F_2 yields in 1950 tests	Number of selections								Mean yield of selections grams/plot
	-5	-4	-3	-2	-1	0	1	2	
5 highest yielding crosses				2	6	18	22	3	225.8
5 next highest yielding crosses			1	8	19	18	5	1	198.2
5 next highest yielding crosses		1	2	4	18	24	3		197.1
5 lowest yielding crosses			7	9	19	9	1		176.9
Grouped according to order of average F_2-F_5 bulk population yields in 1950 tests									
5 highest yielding crosses				3	10	18	19	1	218.9
5 next highest yielding crosses			1	2	14	24	9	3	212.5
5 next highest yielding crosses			2	8	17	16	3		190.2
5 lowest yielding crosses		1	7	10	21	11			176.4

*Mean yield of check varieties = 230.0 grams; standard error = 30.06 grams.

Yields of selections were somewhat influenced by the parental type. The three crosses of six-row awned x two row awned and the four crosses of six-row awned x six row hooded all were low in yield, both as selections and in bulk populations. However, among the 13 crosses of six-row awned parents there also was a marked trend for those crosses superior in yield in bulk populations to produce more high-yielding selections, although differences were not as pronounced as when all 20 crosses are considered.

DISCUSSION

The locations used for this study did not provide as widely different seasonal conditions as those reported for other studies of mixed populations (1, 8). This may make the data somewhat more applicable to the small grain breeding situation at many state experiment stations where most of the breeding and selection work is done at a main station and selections tested later for yield at outlying stations.

Yields from the Shenandoah-produced seed were considerably lower than those from the other locations. This seed was much lighter in weight and it appears that the lower yields from seed produced at Shenandoah can be accounted for primarily by differences in seed quality with inherent yield differences, if present, obscured by other factors.

If yields from Shenandoah-grown seed are omitted, as in the ACM partitions, there still are highly significant differences in yield among seed produced at the other three locations. Only a part of the difference can be accounted for by differences in seed weight. The correlation of .143 between seed weight and yield for the three locations other than Shenandoah, although positive, is not significant. It thus appears that

seed weight was not a major factor influencing yield of seed from Ames, Cresco, and Marcus.

High yields for the F_4 generation produced at Ames may have been somewhat influenced by greater seed weight. Both yield and quality of the F_4 seed produced at Ames in 1948 were high. It seems likely that under these conditions selection for types capable of high yield in a favorable barley season took place and was able to express itself in the favorable 1950 season.

Differential selection for maturity at the different locations also was evident. Populations from the different locations, after one to three generations of natural selection, shifted toward the types favored at these locations. In addition to the differences in yield potential, later-maturing types were favored at both Cresco and Marcus than at Ames and Shenandoah. This is in accordance with expectations, as the locations in northern Iowa are not as likely to have a period of hot, dry weather after heading that would result in a selective advantage for the early-maturing types.

Interaction patterns exhibited between locations, generations, and crosses were of considerable interest. It is apparent that the amount and direction of change in maturity, plant height, and yield was affected considerably by the particular seasonal conditions during the segregating generations. Shifts in composition of the population took place in the generation in which there was a selection pressure, favoring or selecting against a given type at a particular location. None of the locations x crosses or generations x crosses were significant when evaluated within a single test in the same year. It thus appears that the changes in the populations from generation to generation and at the different locations have been essentially alike for this group of crosses, and that bulk populations with considerably different genotypes responded in a similar manner to the selection pressures to which they were subjected.

Bulk yields of the crosses in this investigation gave a satisfactory evaluation of crosses that would be most likely to produce high-yielding selections. This is in agreement with previous results reported for wheat and barley (9, 10, 12) and somewhat at variance with other results presented for oats, barley, wheat, and soybeans (3, 6, 22). From this investigation and others reported in the literature, it appears that yields of bulk crosses have greatest value for predicting the performance of selections from them when the bulk populations are not subject to major changes due to strong selection pressure. If diseases or the length of growing season bring about such major changes, then bulk yields in the early segregating generations are not likely to be representative of the yields of selections made later from the populations. However, if the segregating bulk generations are grown in a series of years that do not depart drastically from normal, information obtained from the bulk population trials may be of considerable value in predicting performance of selections made therefrom.

The relationship observed between the yield of bulk crosses in this study and the number of superior selections from them indicates need for extensive trials to properly evaluate crosses. This agrees with the suggestion of Immer (12) that the evaluation of yield by early generation bulk trials is more accurate if tests are conducted at several locations and for more than one season.

SUMMARY AND CONCLUSIONS

Bulk populations of 20 barley crosses were grown for one to three generations at four locations – near Shenandoah, Ames, Marcus, and Cresco, Iowa. These bulk populations were grown together in tests at Ames and Cresco in 1950 and evaluated for grain yield, plant height, and maturity. Yield tests of selections made from the 20 crosses were grown at Ames and Cresco during the 1950 season.

The location at which the segregating generations had been grown exerted a significant effect upon subsequent bulk population yields, while plant height was not influenced by the varying environments. Date of maturity differed markedly with the location at which segregating generations were grown. Natural selection was for earlier maturity at Ames and Shenandoah and for later types in northern Iowa.

Weight of seed sown was significantly associated with yields in the bulk hybrid tests. However, crosses that were high yielding in the bulk hybrid tests tended to produce the greatest proportion of high-yielding selections. Yield data from the bulk populations in this study could have been used as a basis for discarding entire crosses without appreciable loss of desirable germ plasm for yield.

The predictive value of bulk hybrid yield tests appears to be dependent upon location of the tests and magnitude of selection pressures during the years of test. Wide fluctuations in disease or climatic conditions during the segregating generations tend to make bulk tests unreliable as a basis for selection. Bulk tests, however, may be of considerable value for prediction purposes if conducted through several seasons comparable for plant growth and severity of major plant diseases.

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